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THE UNIVERSITY OF ALBERTA

ENUMERATION AND IDENTIFICATION OF *ENTEROBACTERIACEAE* IN GROUND MEATS

by

Ng, Lai-King

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Enumeration and Identification of Enterobacteriaceae in Ground Meats, submitted by Ng, Lai-King in partial fulfilment of the requirements for the degree of Master of Science.

Michael E. Stiles
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Supervisor

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Date: November 1, 1976

ABSTRACT

The Health Protection Branch of Canada Health and Welfare is proposing amendments to the Canadian Food and Drug Act that would introduce bacterial standards for ground meats. The proposed standards include limits for Escherichia coli, determined by the Most Probable Number technique and the elevated temperature test. This is a time consuming method, which is not suitable for routine quality control purposes. This study compared different methods of enumerating Enterobacteriaceae, coliform bacteria and E. coli in ground meats, using Violet Red Bile agar (VRBA), Mossel's Enterobacteriaceae medium (VRBA + 1% glucose, VRBG) and Most Probable Number techniques using Lauryl Tryptose broth (LST), Brilliant Green Bile 2% broth (BGB), Levine's EMB agar and EC medium. The counts obtained by these different methods and the types of organisms included in the counts were compared.

Of the 169 samples of ground beef included in the study, 62% fell within the proposed E. coli standard of less than 100 per gram, 27% had counts between 100 and 500 E. coli per gram, and 11% exceeded 500 per gram. The relationship between E. coli, coliform and Enterobacteriaceae counts was such that direct plating techniques (VRBA and VRBG) could not be used to estimate E. coli concentrations. However, based on the results of this study, VRBA and EC medium at elevated temperature could be used to give more

rapid results suitable for use in routine quality control.

The bile precipitating colonies isolated from VRBA were primarily E. coli, E. agglomerans and S. liquefaciens, whereas the typical "coliform" colonies growing on EMB were primarily E. coli, C. freundii, K. pneumoniae and E. cloacae. When the isolates from all selective media were re-plated onto VRBA plates, over 50% of E. coli, C. freundii, K. pneumoniae, K. ozaenae and E. cloacae isolates grew on VRBA as bile precipitating (coliform) colonies. On EMB, over 50% of E. coli., C. freundii, K. pneumoniae and E. cloacae grew as typical "coliforms". In addition, other Enterobacteriaceae were frequently present in the ground meats, including E. agglomerans and S. liquefaciens.

In EC medium at 44.5°C and 24h incubation, the majority (92.2%) of organisms producing gas were E. coli type I (IMViC ++--). Increasing the temperature to 45.5°C, decreased the number of false positive E. coli results, but created the need to incubate the samples for 48h instead of 24h.

The BBL Minitek technique, using the biochemical tests selected for this study, proved to be satisfactory for identifying Enterobacteriaceae isolates from ground meats.

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TABLE OF CONTENTS

INTRODUCTION 1

Objectives of Study 3

LITERATURE REVIEW 4
I. The family Enterobacteriaceae 4
II. The Coliform Bacteria 17
A. Classification and Characterization of Coliform Bacteria 18
B. Taxonomy and Nomenclature of Coliform Bacteria 21
C. Atypical Coliforms 21
D. Significance of Coliform Bacteria in Water and Foods 23

III. Development of Media 24

METHODOLOGY 33

I. Coliforms and Enterobacteriaceae Counts in Ground Meats 33
A. Enumeration using the Direct Plating Technique 33
1. Violet Red Bile Agar (VRBA) 33
2. Mossel's Modified VRBA (VRBG) 33
B. Most Probable Number Technique 34
1. Presumptive Test for Coliforms 34
2. Confirmed Test for Coliforms 34
3. Completed Test for Coliforms 34
4. Elevated Temperature Test 34

II. Determination of the Types of Enterobacteriaceae Enumerated by the Different Techniques 35
A. Selection and Purification of Cultures 35
B. Identification of Cultures 36
1. Identification Tests 36
2. Bacteriology 38
3. Tests used to Identify Cultures 38

METHOD DEVELOPMENT 39

I. Comparison of Identification Systems 40
II. Comparison of Enterotube and Modified Minitek Systems to Identify Known Cultures 41
III. Evaluation of Computer Program to Identify Unknown Cultures 47
IV. Selection of Biochemical Methods 49

RESULTS 50

I. Distribution of E. coli, Coliform and Enterobacteriaceae Counts 50

II. Comparison of Counting Techniques 50
A. Differences between Sample Types 50
B. Differences between Media 53
C. Correlation between Different Counts 54
D. Effect of Temperature and Incubation Time on VRBA and VRBG 56
E. EC Test 56
1. Comparison of the Direct and Indirect EC Tests 56
2. Comparison of EC Tests at 44.5 and 45.5°C 57

III. Identification of Enterobacteriaceae Isolates 59
A. Confirmation of Colony Morphology 59
B. Association of Colony Morphology with Types of Organisms 61
1. Violet Red Bile Agar: Coliform Medium ... 63
2. Mossel's Violet Red Bile Glucose agar: Enterobacteriaceae Medium 65
3. Eosin Methylene Blue agar: Confirmed Coliforms By MPN Technique 67
C. Interrelationships of Colony Morphology between the Selective Media 69
D. Growth Characteristics of Enterobacteriaceae Isolates in Lauryl Tryptose broth (LST) and Lactose broth 75
E. Relationship of Enterobacteriaceae Isolates with IMViC Types 80
F. Association of Enterobacteriaceae with Elevated Temperature Test Results 83

IV. Evaluation of Minitek Technique for Identification of Enterobacteriaceae from Meat Samples .. 85

DISCUSSION AND CONCLUSIONS 88

I. Coliform and Enterobacteriaceae Counts 88
II. Classification of Coliform Bacteria 96
III. Minitek Identification Technique 98

SUMMARY 102

BIBLIOGRAPHY 104

APPENDIX A. Identification of 10 Known and 7 Unknown Enterobacteriaceae Cultures using Different Identification Systems 115

APPENDIX B. Results for Identification of ATCC Type Cultures 116

APPENDICES A and B: Addendum 117

LIST OF TABLES

Table 1. The habitat, and sources of members of the family Enterobacteriaceae 6

Table 2. Tribes and genera of the family Enterobacteriaceae from the 5th to 8th editions of Bergey's Manual 8

Table 3. Relationship of the tribe Escherichieae in the 8th edition of Bergey's Manual with Escherichieae and Salmonelleae in previous editions 9

Table 4. Relationship of the tribe Klebsielleae in the 8th edition of Bergey's Manual with Escherichieae and Serrateae in previous editions 11

Table 5. Relationship of the tribe Proteeae and Yersinieae in the 8th edition of Bergey's Manual with Proteeae in previous editions 14

Table 6. Relationship of the tribe Erwineae in the 8th edition of the Bergey's Manual with Erwineae in the previous editions 15

Table 7. Distribution of Enterobacteriaceae count of ground beef 51

Table 8. Statistical analysis of the E. coli, coliform, and Enterobacteriaceae counts on raw ground meats 52

Table 9. Pearson's correlation coefficients between 8 different Enterobacteriaceae, coliform and E. coli counts 55

Table 10. Relationship between elevated temperature tests in EC broth at 44.5°C and 45.5°C 58

Table 11. Comparison of morphology of colonies originally isolated from Violet Red Bile agar (VRBA) with their morphology when re-plated on VRBA after purification 60

Table 12. Comparison of morphology of colonies originally isolated from Violet Red Bile agar + 1% glucose (VRBG) with their morphology when re-plated on VRBG after purification 60

Table 13. Comparison of morphology of colonies originally isolated from EMB agar with their morphology when re-plated on EMB after purification 62

Table 14. Identity and number of organisms associated with different colony types on Violet Red Bile agar (VRBA) from original isolates and for purified (re-plated) cultures 64

Table 15. Identity and number of organisms associated with different colony types on Mossel's Enterobacteriaceae medium (VRBG) form original isolates and for purified (re-plated) cultures 66

Table 16. Identity and number of organisms associated with different colony types on Levine EMB agar from original isolates and for purified (re-plated) cultures 68

Table 17. Growth characteristics of Enterobacteriaceae isolates on Violet Red Bile agar (VRBA) 71

Table 18. Growth characteristics of Enterobacteriaceae isolates on Mossel's Violet Red Bile agar + 1% glucose (VRBG) 72

Table 19. Growth characteristics of Enterobacteriaceae isolates on Levine EMB agar 74

Table 20. Growth characteristics of Enterobacteriaceae isolates in Lauryl Sulphate Tryptose broth (LST) 76

Table 21. Growth characteristics of Enterobacteriaceae isolates in Lactose broth 77

Table 22. Comparison of growth characteristics of Enterobacteriaceae isolates in Lauryl Sulphate Tryptose (LST) and Lactose broths 79

Table 23. Occurrence of IMViC types within Enterobacteriaceae isolates with different Lauryl Tryptose broth (LST) and Lactose reactions 79

Table 24. Relationship of lactose positive organisms and IMViC types 81

Table 25. Relationship of lactose negative organisms and IMViC types 82

Table 26. Distribution of organisms with different growth characteristics in EC broth at 44.5°C 84

Table 27. Comparison of glucose and lactose fermentation in Triple Sugar Iron (TSI) slant and Phenol Red broth tubes 86

LIST OF FIGURES

Figure 1. Flow diagram illustrating the tests used to differentiate the phenylalanine deaminase producing Enterobacteriaceae 43

Figure 2. Flow diagram illustrating the tests used to differentiate the phenylalanine deaminase lacking Enterobacteriaceae which produce H_2S 44

Figure 3. Flow diagram illustrating the tests used to differentiate the phenylalanine deaminase lacking, indole positive and H S negative Enterobacteriaceae 45

Figure 4. Flow diagram illustrating the tests used to differentiate the phenylalanine deaminase lacking, indole and H_2S negative Enterobacteriaceae 46

INTRODUCTION

Among the coliforms, *Escherichia coli* has become the predominant indicator of faecal contamination in food and water supplies, because it is a commensal organism of the intestinal tract of humans and other warm blooded animals. Since most *E. coli* are harmless to man, their importance lies in their indication of the possibility that enteric pathogens might be present. *E. coli* has a relatively short survival time in water compared to other faecal indicator organisms (such as faecal streptococci and *Clostridium perfringens*), so Public Health Authorities view the presence of *E. coli* in water as an indication of recent faecal contamination.

The significance of *E. coli* in foods is somewhat different. *E. coli* can survive on equipment and in food, and their presence does not necessarily indicate either direct or recent faecal contamination. However, if *E. coli* of faecal origin is present, enteric pathogens might survive with them. Although unhygienic handling of foods is a possible source of contamination, inadequate sanitation of equipment represents their most probable source. Organisms on equipment are from various sources, therefore other coliforms, besides *E. coli*, are also emphasized to give a wider and more accurate indication of sanitation and hygiene.

Coliforms are usually present in raw milk, therefore their presence in pasteurized milk and dairy products would

indicate inadequate pasteurization or post-pasteurization contamination. The use of coliforms to indicate potential health hazards in all foods has been criticized, since some foods contain non-lactose fermenting Enterobacteriaceae, which are more significant to public health than coliforms. Some workers suggest enumeration of Enterobacteriaceae as an indicator of safety (Buttiaux and Mossel, 1961; Mossel, 1957; Mossel, et al., 1962, 1963).

To enumerate coliforms in water, the American Public Health Association (APHA, 1965, 1971) recommended the Most Probable Number (MPN) technique. Lactose or Lauryl Sulfate Tryptose broths are used as a presumptive test, Brilliant Green Bile 2% broth as a confirmed test and Eosin Methylene Blue agar or Endo agar for the completed test. Subsequently, faecal and non-faecal strains must be differentiated. The faecal E. coli can be differentiated from other coliforms by IMViC and/or elevated temperature tests.

In dairy products, since the emphasis is on more rapid enumeration of coliforms (lactose-fermenting Enterobacteriaceae) solid media such as Violet Red Bile agar (VRBA) and Desoxycholate Lactose agar have been developed. The use of these solid media has been employed for enumeration of coliforms in other foods. Modification of Violet Red Bile agar by addition of 1% glucose gives a medium on which Enterobacteriaceae can be enumerated (Mossel, 1957; Mossel et al., 1962; Mossel and Ratto, 1970; Thatcher and Clark,

1968).

Atypical colonies have been observed on Violet Red Bile agar. These atypical colonies include coliform and non-coliform bacteria. When VRBA was used to enumerate coliforms in meats, large numbers of atypical, non-bile precipitating colonies were observed (Stiles, 1973). Whether these colonies should be included in the counts is questionable, because the identity of the colonies was not known.

Objectives of Study

The objectives of this study were to determine the types of Enterobacteriaceae present in ground meats, and the influence of the method of enumeration on the types of Enterobacteriaceae included in the counts.

The study was also planned to evaluate the use of IMViC typing and the elevated temperature test for classification of coliforms in general, and faecal E. coli, in particular.

The study employed the Minitek system for identification of Enterobacteriaceae, and this identification system will be evaluated.

LITERATURE REVIEW

The Enterobacteriaceae are facultative, anaerobic, Gram negative, nonsporeforming rods that can ferment glucose usually with, but sometimes without, gas production. They are oxidase negative, can reduce nitrate to nitrite, and, if motile, they are peritrichously flagellated (Bergey's Manual, 1974). They are commonly found as intestinal organisms, and as a result they are frequently referred to as the enteric bacteria. Within the Enterobacteriaceae are the "coliform" bacteria. These organisms are of considerable importance in water and food microbiology, so they are considered in this review as a separate entity.

I. The family ENTEROBACTERIACEAE

The Enterobacteriaceae are often referred to as the "Coli-Typhoid" group, but not all members of this family are intestinal parasites or human pathogens. Typical sources and habitats of the members of this family, as well as their pathogenicity are given in Bergey's Manual and are summarized in Table 1. E. coli is generally regarded as a nonpathogenic, commensal intestinal organism. However, some strains are enteropathogenic (E.E.C.) causing enteritis in infants and adults.

The most common intestinal pathogens among the Enterobacteriaceae are Salmonella and Shigella spp., which cause salmonellosis and shigellosis, respectively. Salmonella are

widespread in Nature and can be found in foods and water. The ubiquitous nature of Salmonella has developed from their endless ecocycle (Taylor and McCoy, 1969). Shigella, on the other hand, are obligate parasites of humans and monkeys and are seldom associated with foods. Shigelloses generally result from drinking contaminated water or contact with infected persons.

In addition to these intestinal pathogens, other genera and species of the Enterobacteriaceae have been implicated as pathogens, but they are not generally considered to be "food poisoning" organisms. For example, Proteus spp., Citrobacter spp., Klebsiella spp. and Yersinia spp. are considered to be pathogenic. Edwardsiella tarda, although not usually considered a food poisoning microorganism, has been associated with outbreaks of diarrhoea (Bryan, 1969). The Erwinia spp. are plant pathogens and have not been implicated in animal diseases.

The organisms in Table 1 are listed according to the 8th edition of Bergey's Manual. There are marked differences in taxonomy and nomenclature of the Enterobacteriaceae between the 8th and the previous editions of Bergey's Manual. As a result, there could be difficulties in interpreting the literature on Enterobacteriaceae, for example E. coli used to describe lactose-fermenting, aerogenic strains; now non-lactose fermenting, anaerogenic strains such as the Alkalescens-Dispar group and

Table 1. The habitat, and sources of members of the family Enterobacteriaceae, based on Bergey's Manual (1974)

Organisms	Habitat and Sources
E. coli	Intestine of warm blooded animals
E. tarda	Intestinal tract of snakes, human faeces and water
Citrobacter spp.	Water, food, faeces and urine
Salmonella spp.	Intestinal tract of warm-blooded animals and reptiles, and food
Shigella spp.	Intestine of man and higher monkeys
K. pneumoniae	Soil, water, grain and intestinal tract of man and animals
K. ozaenae	Infections of respiratory tract
K. rhinoscleromatis	Rhinoscleroma disease
E. cloacae	Faeces of man and animals, sewage, soil and water
E. aerogenes	Faeces of man and animals, sewage, soil, water and dairy products
S. marcescens	Water, soil and food
P. vulgaris P. mirabilis	Faeces of animals, sewage, soil, especially with protein decomposition
P. morganii	Faeces
P. rettgeri	Chicken faeces, clinical specimens
P. inconstans	Urine and faeces
Y. pestis	Buboes, blood, sputum and lung exudate
Y. pseudotuberculosis	Pseudotuberculosis lesions, intestinal tract
Y. enterocolitica	Faeces, lymph nodes, milk and ice cream
Erwinia spp.	Plant pathogens, saprophytes or epiphytic flora

Paracolobactrum sp. have been included. The purpose of this section is to indicate the relationship between the Tribes, Genera and species of Enterobacteriaceae in the 8th edition of Bergey's Manual and those in the 7th, 6th and 5th editions (Bergey's Manual, 1939, 1948, 1957, 1974). The Tribe and Genus classifications used in these editions of the Manual are shown in Table 2.

Tribe I. Escherichieae. Consists of 5 genera in the 8th edition. The relationship between these genera in the 8th edition and those of the previous 3 editions are shown in Table 3.

The genus Escherichia in the 8th edition of Bergey's Manual has only one species: E. coli. In previous editions there were other species of this genus. Of these, only the pigmented E. aurescens has been incorporated into E. coli, the other species have been assigned to the genus Citrobacter. However, it appears that some Shigella species, S. alkalescens and S. dispar of the Alkalescens-Dispar group, have now been incorporated with E. coli. In addition, slow and non-lactose fermenting Paracolobactrum coliforme of the 7th edition, are also included in E. coli. These changes have broadened the biochemical characteristics of E. coli to include anaerogenic and non-lactose fermenting strains.

The other Escherichia species, E. freundii and E. intermedia(um), have been incorporated into the genus Citrobacter, as C. freundii and C. intermedius, respectively.

Table 2. Tribes and genera of the family Enterobacteriaceae from the 5th to 8th editions of Bergey's Manual

Tribe	Genera		
	5th edition	6th edition	7th edition
Escherichieae (iae)	Escherichia Aerobacter Klebsiella	Escherichia Aerobacter Klebsiella Paracolobactrum	Escherichia Aerobacter Klebsiella Paracolobactrum Alginobacter
Erwineae	Erwinia	Erwinia	Erwinia
Serrateae	Serratia	Serratia	Serratia
Proteae	Proteus	Proteus	Proteus
Salmonelleae	Salmonella Eberthella Shigella	Salmonella Shigella	Salmonella Shigella

Tribe	8th edition
Escherichieae	Escherichia Edwardsiella Citrobacter Salmonella Shigella
Klebsielleae	Klebsiella Enterobacter Hafnia Serratia
Proteae	Proteus
Yersinieae	Yersinia
Erwineae	Erwinia

Table 3. Relationship of the tribe Escherichieae in the 8th edition of Bergey's Manual with Escherichieae and Salmonelleae in previous editions

8th edition	7th edition	6th edition	5th edition
Genus I: *Escherichia*			
E. coli	*E. coli*	*E. coli*	*E. coli*
	S. alkalescens	*S. alkalescens*	*S. alkalescens*
	E. aurescens		
	P. coliforme		
Genus II: *Edwardsiella*			
E. tarda	-	-	-
Genus III: *Citrobacter*			
C. freundii	*E. freundii*	*E. freundii*	*E. freundii*
C. intermedius	*E. intermedia*	*E. intermedium*	-
Genus IV: *Salmonella*			
No significant changes between editions			
Genus V: *Shigella*			
S. sonnei	*S. sonnei*	*S. sonnei*	*S. sonnei*
		S. ceylonensis	*S. ceylonensis*
S. flexneri	*S. flexneri*	*S. paradysenteriae*	*S. paradysenteriae*
S. boydii	*S. boydii*	-	-
S. dysenteriae	*S. dysenteriae*	*S. dysenteriae*	*S. dysenteriae*
	S. schmitzii	*S. ambigua*	*S. ambigua*
	S. arabinotarda		

- No cross reference of this nomenclature to organisms in the 7th, 6th or 5th editions.

Other genera added to the Tribe Escherichieae in the 8th edition include: Edwardsiella, Salmonella and Shigella. The genus Edwardsiella has only one species, E. tarda. The association of Edwardsiella with organisms in previous editions is not clear. Salmonella and Shigella were previously in the Tribe Salmonelleae, but this tribe has been eliminated from the classification in the 8th edition.

The main emphasis in classifying both Salmonella and Shigella in the 8th edition is their antigenic structure. However, the biochemical characteristics have not been completely eliminated at the species level. Most of the species that are still described according to their biochemical reactions in the 8th edition have been carried forward from previous editions. The genus Eberthella mentioned in the 6th edition has been included with Salmonella in the 7th and 8th editions.

Tribe II. Klebsielleae. This tribe is new to the classification, and consists of 4 genera. The relationship between these genera and those of previous editions is shown in Table 4. The tribe Klebsielleae consists of some organisms from the Escherichieae in previous editions, as well as from the tribe Serrateae.

The genus Klebsiella has 3 species: K. pneumoniae, K. ozaenae and K. rhinoscleromatis. In the 6th edition, the latter two species were classified as strains of K. pneumoniae, however in the other editions these organisms

Table 4. Relationship of the tribe Klebsielleae in the 8th edition of Bergey's Manual with Escherichieae and Serrateae in previous editions

8th edition	7th edition	6th edition	5th edition
GENUS I: *Klebsiella*			
K. pneumoniae	*K. pneumoniae* *A. aerogenes* (non-motile) *P. aerogenoides*	*K. pneumoniae* *A. aerogenes* (non-motile)	*K. pneumoniae* *A. aerogenes* (non-motile) *K. granulomatis* *K. paralytica* *K. capsulata*
K. ozaenae *K. rhinoscleromatis*	*K. ozaenae* *K. rhinoscleromatis*	*K. pneumoniae* *K. pneumoniae*	*K. ozaenae* *K. rhinoscleromatis*
GENUS II: *Enterobacter*			
E. cloacae *E. aerogenes*	*A. cloacae* *A. aerogenes* (motile)	*A. cloacae* *A. aerogenes* (motile)	*A. cloacae* *A. aerogenes* (motile)
GENUS III: *Hafnia*			
H. alvei	-	-	-
GENUS IV: *Serratia*			
S. marcescens	*S. marcescens* *S. plymuthica* *S. kiliensis* *S. piscatorum* *S. indica*	*S. marcescens* *S. plymuthicum* *S. kiliensis* *S. piscatorum* *S. indica*	*S. marcescens* *S. plymouthensis* *S. kilensis* *S. piscatora* *S. indica* *S. anolium*

- No cross reference of this nomenclature to organisms in the 7th, 6th or 5th editions.

were separate species. K. pneumoniae has been expanded to include the non-motile strains of Aerobacter aerogenes (7th, 6th and 5th editions), Paracolobactrum aerogenoides (7th edition) and other Klebsiella spp. (5th edition).

The genus Enterobacter was primarily derived from the previous Aerobacter, and consists of two species: E. cloacae and E. aerogenes. E. aerogenes consists of only the motile strains of the previous A. aerogenes. Enterobacter agglomerans, described by Ewing and Fife (1972) is placed in the Herbicola group as Erwinia herbicola in the current edition of Bergey's Manual, and is discussed later.

The genus Hafnia consists of one species: H. alvei. Furthermore, there are some references to E. alvei (Bergey's Manual, 1974) and E. hafniae (Roche Encise II[1]) in the literature, but these names are not used within the family Enterobacteriaceae in any of the editions of Bergey's Manual. The association between this organism in the 8th edition and those in the other editions is not specified.

The genus Serratia also consists of only one species: S. marcescens, and includes all of the Serratia spp. in previous editions. Some species are recommended for addition to this genus, and will be discussed later.

---

[1] The Encise II (Enterobacteriaceae Numerical Coding and Identification System for Enterotube) was developed by Roche Diagnostic, Professional Services Development, 340 Kingsland Street, Nutley, N.J., U.S.A.

Tribe III. Proteeae. This tribe consists of one genus with 5 species. There has been little change in this group between editions, as shown in Table 5.

Tribe IV. Yersinieae. This tribe is introduced in the 8th edition for the first time. There is one genus Yersinia, which was derived from Pasteurella in previous editions and now consists of 3 species, as shown in Table 5.

Tribe V. Erwineae. This has the one genus Erwinia. Some species have been added or eliminated between editions of the Manual (see Table 6).

Some organisms listed in the previous editions of Bergey's Manual were not included in Tables 3-6, because there is insufficient information to show how they were related to organisms in the new taxonomic scheme of the 8th edition. These organisms include Alginobacter sp. (7th ed.); several Erwinia spp. (6th ed.); Proteus hydrophilus, Proteus ichthyosmus, Erwinia flavida and Erwinia erivanesis (5th ed.). According to the 8th edition, Erwinia mimipressualis and Erwinia dissolvans should be placed under the genera Enterobacter and Klebsiella, respectively, however the exact nomenclature of these organisms under these genera is not indicated.

In 1972 , Ewing and Fife described an organism named E. agglomerans. This organism includes the Herbicola-Lathyri bacteria which have been retained in the genus Erwinia in

Table 5. Relationship of the tribe *Proteeae* and *Yersinieae* in the 8th edition of Bergey's Manual with *Proteeae* in previous editions

8th edition	7th edition	6th edition	5th edition
TRIBE: *Proteeae*			
GENUS I: *Proteus*			
P. vulgaris	*P. vulgaris*	*P. vulgaris*	*P. vulgaris*
P. mirabilis	*P. mirabilis*	*P. mirabilis*	*P. mirabilis*
			P. americanus
			P. pseudovalerieiei
			P. ammoniae
P. morganii	*P. morganii*	*P. morganii*	*P. morganii*
P. rettgeri	*P. rettgeri*	*P. rettgeri*	*S. rettgeri*
P. inconstans	*P. inconstans*	-	-
TRIBE: *Yersinieae*			
GENUS I: *Yersinia*			
Y. pestis	-	-	-
Y. pseudo-tuberculosis	-	-	-
Y. enterocolitica	-	-	-

- No cross reference of this nomenclature to organisms in the 7th, 6th or 5th edition.

Table 6. Relationship of the tribe Erwineae in the 8th edition of the Bergey's Manual with Erwineae in the previous editions

8th edition	7th edition	6th edition	5th edition
GENUS I: Erwinia			
Species:			
amylorova	amylorova	amylorova	amylorova
salicis	salicis	salicis	salicis
tarcheiphila	tarcheiphilla	tarcheiphila	tarcheiphila
nigrifluens	-	-	-
quercina	-	-	-
rubrifaciens	-	-	-
herbicola	vitivora	vitivora	-
	milletiae	milletiae	milletiae
	cassavae	cassavae	cassavae
	ananas	ananas	ananas
	citrimaculans	citrimaculans	citrimaculans
	mangiferae	mangiferae	manganiferae
		lathyri	lathyri
stewartii	-	-	-
uredovora	-	-	-
carotovora	carotovora	carotovora	carotovara
	carnegieana	carnegieana	solanisapra
	atrospetica	atroseptica	phytophthora
	aroideae	cytolytica	aroideae
	arsideae		
chrysanthemi	chrysanthemi	-	-
cypripedii	-	-	-
rhapontici	rhapontici	rhapontici	-

- No cross reference of this nomenclature to organisms in the 7th, 6th or 5th editions.

the 8th edition of Bergey's Manual. E. agglomerans was shown not to be a strict plant pathogen, in that it has been isolated from clinical specimens of humans and lower animals. This organism forms chromogenic (yellow) colonies and consists of aerogenic and anaerogenic strains. The authors placed this organism under the genus Enterobacter instead of in a new genus because of its similarity to other Enterobacter spp. E. agglomerans is used to describe this organism in this text, because it is more readily associated with this name in identification procedures.

Ewing et al. (1973) described Serratia liquefaciens (E. liquefaciens) and Serratia rubidaea. This increases the species to 3, in the genus Serratia. These organisms have been isolated from human clinical and intestinal specimens, animals and foods. Almost 50% of 109 isolates of S. liquefaciens were obtained from foods, and most of these were dairy products. Bergey's Manual is not clear on the designation of S. liquefaciens, referring to E. liquefaciens as non-pigmented Serratia sp. In this text, this organism is referred to as S. liquefaciens as opposed to an Enterobacter.

Levinea has been proposed as a new genus of the family Enterobacteriaceae by Young et al. (1971) because of its unique biochemical and serological properties. Two species are proposed, which have been isolated from human clinical and faecal samples. Although these organisms appear to be

closely related to Citrobacter and Enterobacter, they can be distinguished by biochemical differences.

II. The Coliform Bacteria

The term "coliforms" is widely used by bacteriologists, sometimes without strict regard for the definition. Furthermore, there are many definitions of coliforms, depending on the method and purpose of their detection and enumeration. According to the Standard Methods for Water and Sewage analysis (APHA, 1971), coliforms are defined (using liquid media) as "the aerobic and facultative anaerobic, Gram negative, nonsporeforming rod-shaped bacteria which ferment lactose with gas production within 48h at 35°C." The coliform group, thus defined, is equivalent to previously used descriptions, such as: B. coli, coli-aerogenes and the colon group (APHA, 1965).

An alternate definition used by water bacteriologists is based on the membrane filter technique, in which coliforms are defined as "those organisms that produce dark colonies with metallic sheen within 24h on an Endo-type medium containing lactose" (APHA, 1971). In dairy bacteriology, when coliform counts are high, they are usually enumerated on Violet Red Bile agar (VRBA) or Desoxycholate agar (APHA, 1967). On VRBA, the coliforms grow as typical dark red colonies, surrounded by a zone of bile precipitation, within 24h incubation at 32°C (APHA, 1967).

Although there are many definitions for the coliforms, they are all based on the fact that these organisms ferment lactose and can grow in the presence of bile salts at 32-37°C. Since there have been extensive taxonomic changes in the family Enterobacteriaceae, the names of the organisms included within the coliforms have varied.

A. Classification and Characterization of Coliform Bacteria

Lactose was originally selected as the basis of differentiating between the commensal intestinal organisms and those with pathogenic implications (Winslow et al., 1919). The lactose fermenting bacteria in this group were further divided into those that were typical intestinal parasites (B. coli) and those that were most frequently isolated from grass, grains and soil (A. aerogenes) (MacConkey, 1905; Rogers, 1918). The classification and characterization of the coliforms was greatly influenced by the practical considerations of water bacteriologists, who wanted to identify intestinal coliforms as a sanitary index (Parr, 1939; Rogers et al., 1918; Winslow et al., 1919).

There were many attempts to relate types of coliforms and their biochemical characteristics to the ecological source of the organisms (Bardsley, 1934; Rogers et al., 1914, Rogers et al., 1918; Winslow et al., 1919). Carbohydrate fermentation was first used by MacConkey (1905) as a means of differentiating lactose fermenters from

various ecological sources. He used sucrose and dulcitol to divide the lactose fermenters into 4 groups. These groups were further subdivided using gelatin liquefaction and the Voges-Proskauer reaction as the basis of differentiation. Other studies failed to give a system for classifying the source of coliforms using carbohydrates alone and Parr (1939), established a series of tests for classifying coliforms:

Indole production
Methyl Red test
Voges-Proskauer reaction
Citrate utilization
Uric acid utilization
Gelatin liquefaction
Eijkman elevated temperature test
H_2S production
Sucrose and inositol fermentation
Alpha-methyl-d-glucoside fermentation

It had been reported (Bardsley, 1934; Levine, 1918), that the lactose fermenting bacilli could be divided into two ecological groups based on their Methyl Red (MR) and Voges-Proskauer (VP) reactions. The coli group (E. coli) of intestinal origin were MR+, VP-, while the aerogenes-cloacae group from the soil were MR-, VP+. Using these two criteria alone was found to be inadequate because other combinations of MR-VP reactions were observed. As a result Koser's citrate reaction and the indole test were added to the classification.

Parr noted that Indole, Methyl Red, Voges-Proskauer and the Citrate tests (IMViC tests) were those most frequently

used to differentiate the "faecal" and "non-faecal" coliforms (Parr, 1939). *E. coli* (IMViC ++--) was shown to be the most typical, numerous and constant coliform organism found in faeces, and therefore most useful as a sanitary index for water (Bardsley, 1934).

The ability of *E. coli* to produce gas at elevated temperatures (46°C) was established by Eijkman (1904). In 1949, the Coliform Sub-Committee of the Society of Applied Bacteriologists (Report, 1949) proposed the classification of the lactose fermenting coli-aerogenes bacteria based on the IMViC tests and ability to produce gas from lactose in MacConkey broth at 44°C. This classification was revised by the Coliform Sub-Committee in 1956, using gelatin liquefaction as an additional characteristic, as follows:

Coli-aerogenes Organism (1956 Classification)	I	M	ViC	44°C	Gelatin
E. coli I	+	+	- -	+	-
E. coli III	+	+	- -	-	-
E. coli II	-	+	- -	-	-
C. freundii I	-	+	- +	-	-
C. freundii II	+	+	- +	-	-
K. aerogenes I	-	-	+ +	-	-
K. cloacae I	-	-	+ +	-	+
E. carotovora	-	-	+ +	-	+
K. aerogenes II	+	-	+ +	-	-

Subsequently, the American Public Health Association (12th edition, 1965) classified coliform bacteria into three groups, based solely on the IMViC tests.

Organism	I	M	Vi	C
E. coli I	+	+	-	-
E. coli II	-	+	-	-
E. freundii I	-	+	-	±
E. freundii II	+	+	-	-
A. aerogenes I	-	-	+	±
A. aerogenes II	±	-	+	+

B. Taxonomy and Nomenclature of Coliform Bacteria

The taxonomy and nomenclature of the Enterobacteriaceae has been greatly influenced by changes in classification of the coliforms, and vice versa, as illustrated by Cowan (1956) when he reviewed the report of the Coli-aerogenes Sub-Committee. Besides the coliforms, the non-lactose fermenters which are of clinical significance have also undergone changes in classification. Both have affected the taxonomy of the Family Enterobacteriaceae as a whole.

C. Atypical Coliforms

Typical coliforms can produce acid and gas from lactose broth within 24h. Atypical coliforms are frequently encountered (Parr, 1939). Some are slow lactose fermenters, that

is, they do not ferment lactose for a considerable number of days. Some atypical forms ferment lactose but fail to produce gas (anaerogenic). Some strains give all of the reactions of Escherichia except the fermentation of lactose, and have been referred to as the "paracoli" (Parr, 1939). Some variants ferment lactose at room temperature but not at 37°C.

Sometimes, coliforms become atypical when grown in adverse environments. For example, sodium benzoate in weak glucose broth retarded the fermentative activities of E. coli, whereas other characteristics were only slightly affected. No gas producers could be detected if foods were preserved with sodium benzoate (Smirnov, 1916). Sodium acetate also inhibits coliforms from producing gas from sugars, though they can ferment alcohol derivatives of the same sugars.

Some variants are unstable, with a variable colony type as well as biochemical activity (Jones et al., 1966; Parr, 1939). There have been many reports of atypical colonies on VRBA (Hartman, 1960; Jones et al., 1966; Kereluk and Gunderson, 1959; Morris and Cerny, 1954; Ross and Thatcher, 1958). In some cases the atypical colonies have been identified as E. coli (Hartman, 1960; Jones et al., 1966) and should be included in the VRBA "coliform" count. In other cases, the atypical, non-bile precipitating colonies have been shown to be other organisms such as "Proteus,

Aerobacter, *Pseudomonas*, paracolon organisms, and *Flavobacterium*" (Ross and Thatcher, 1958). Although some of these organisms might be included in the "coliform" count, the pseudomonads and flavobacteria would give spurious results.

D. Significance of Coliform Bacteria in Water and Foods

In water bacteriology, *E. coli* I was selected as an indicator among the coliform bacteria, because of the specificity of *E. coli* I as an indicator of faecal contamination. In food bacteriology, the presence of coliforms indicates either unhygienic conditions by the presence of *E. coli* I or unsanitary conditions by the presence of other coliform bacteria. According to Buttiaux and Mossel (1961), a suitable indicator of faecal contamination should have the following characteristics:

1. The bacteria selected should occur only in the intestinal environment.
2. They should occur in very high numbers in faeces.
3. They should have a high resistance to the extraenteral environment.
4. They should permit easy and reliable detection even when present in very low numbers.

Besides *E. coli* I, Buttiaux and Mossel (1961) considered that the *Klebsiella* group (non-motile, capsulated, VP+ and urease+) are always of faecal origin,

that the Citrobacter group are not only found in faeces but also in soil, and that the Enterobacter groups are rarely found in the human intestine. In foods that have been dehydrated, frozen or refrigerated, Buttiaux and Mossel (1961) suggested that E. coli may not survive as well as some enteric pathogens that might also be present. As a result, E. coli I might not be the best indicator organism for unhygienic handling of foods. They suggested Klebsiella as an alternate indicator to E. coli and stressed that non-lactose fermenters should not be overlooked e.g. Proteus spp. and non-lactose fermenting E. coli (Paracolobactrum coliforme).

Where routine tests are being conducted on foods for non-lactose fermenting enteric pathogens e.g. Salmonella spp., indicator organisms are of little concern. However, when safety is based on indicator organisms, Buttiaux and Mossel (1961) considered that the Enterobacteriaceae as a whole should be used, in place of the coliform bacteria.

III. Development of Media

The development of solid and liquid media for the enumeration or estimation of Enterobacteriaceae occurred simultaneously. MacConkey (1908), summarized the development of bile salts as a selective agent for "colon bacilli" (Bacillus typhosus and Bacillus coli communis). The medium was intended to be versatile by changing the carbohydrates.

In the case of differentiating B. typhosus and B. coli, lactose was the carbohydrate selected because these organisms grew as bile and non-bile precipitating colonies (MacConkey, 1908). In water and dairy product analysis, lactose also became the carbohydrate of choice, because emphasis was placed on detection of E. coli and lactose fermenters (Coliform bacteria) (Hall and Ellefson, 1918a,b).

Winslow and Dolloff (1922) studied the effect of bile added to Brilliant Green Lactose and Gentian Violet Lactose broths and they recommended the use of bile in the media to reduce the inhibitory effect of the dyes on the coliforms. The use of dyes was introduced by Churchman (1913) and Hall and Ellefson (1918a,b), to eliminate the false results in the presumptive lactose broth tests, due to sporogenous, anaerobic and aerobic, Gram positive bacteria growing in the broth (Hall and Ellefson, 1918a,b). However, it was shown that selective media, such as Brilliant Green Bile broth inhibited some coliform organisms (Mallmann and Darby, 1941; Winslow and Dolloff, 1922). The Standard Method Committee of the American Public Health Association suggested the use of Lactose broth as a presumptive test and Brilliant Green Bile broth as a confirmed test for coliform organisms (Mallmann and Darby, 1941).

Mallmann and Darby (1941) observed that tryptose added to Lactose broth caused many "slow lactose fermenters" to produce gas in greater quantities in a shorter period of

time. Adding lauryl sulfate also gave selection of coliforms. This resulted in the development of Lauryl Sulfate Tryptose broth (Mallmann and Darby, 1941), which was shown to be more reliable for isolation of coliforms than Lactose broth (Hajna and Perry, 1943; Mallmann and Darby, 1941; Perry and Hajna, 1944), and to be a good presumptive medium for use with foods (Hall, 1964).

Other selective broths were also developed for the isolation of coliforms. These included: Formate Ricinoleate broth (Stark and England, 1935), Buffered Desoxycholate Glucose broth (Hajna and Damon, 1955), EC medium (Eijkman, 1904) and its modifications (Hajna, 1937; Perry and Hajna, 1933,1944) and Boric Acid Lactose broth (Clark et al., 1957; Njoku-Obi and Skinner, 1957; Vaughn et al., 1951). The latter two broths, EC medium and Boric Acid Lactose, became involved in the development of elevated temperature tests for the detection of faecal E. coli, which is discussed below (see p. 27).

Salle (1930), summarized the development of selective media for the confirmatory test of E. coli as follows:

"Very few of the media now extensively in use were compounded solely for water examinations. This accounts for the large number of modifications of Endo and other differential media. This is a point that should be kept in mind.

Drigalski and Conradi (1902) used crystal violet to eliminate other organisms interfering with the isolation of B. typhosus from stools. Litmus was added to the medium to identify the presence of the acid producing colon organisms. The well known medium of Endo (1903) is composed of basic fuchsin decclorized by the use of sodium sulfite. Modifications of the original formula have been reported by Kendall

and Walker (1910), Kendall and Day (1911), Kinyoun and Deiter (1912), Harding and Ostenberg (1912), Robinson and Rettger (1916), Levine (1918), and others adapting the medium to special investigations. Holt-Harris and Teague (1916), incorporated the dyes methylene blue and eosin in an agar base containing lactose and sucrose and were able to isolate the typhoid and dysentery organisms on this preparation. This medium has been modified and simplified by Levine (1918 and 1921), adapting its use to water works laboratories."

Subsequently, Bartrum and Black (1936) compared media for the isolation of coliform bacteria. They recommended Violet Red Bile agar, as well as Neutral Red Bile agar and Brilliant Green Bile broth as giving reliable confirmatory tests. Violet Red Bile agar was shown to be satisfactory for direct plating and enumeration of E. coli in dairy products (Miller and Prickett, 1939). Leifson (1935) developed selective media containing sodium desoxycholate as the inhibitory agent. Selectivity was modified by changing the energy source, for example, lactose was used in the medium to isolate coliform bacteria, whereas citrate was used to isolate non-lactose fermenting, intestinal pathogens.

Eijkman (1904) developed the elevated temperature technique to differentiate E. coli from other coliform bacteria. Since then, there have been many modifications of both the medium and incubation temperature. Perry and Hajna (1933) added phosphate buffer, reduced the amount of glucose to 0.3%, and used a pH of 5.6 as opposed to 4.5. Other carbohydrates were also suggested to replace glucose, such as lactose and mannitol (Hajna, 1937). In a comparison of the modified Eijkman lactose broth (EC medium) with

MacConkey broth, the EC medium was shown to be superior (Hajna and Perry, 1939). Another medium used for the elevated temperature test was Boric Acid Lactose broth (Clark et al., 1957; Njoku-Obi and Skinner, 1957; Vaughn et al., 1951). At 43°C, this medium was reported to be selective for E. coli in different foods, especially citrus products (Levine et al., 1955; Njoku-Obi and Skinner, 1957; Walford, 1954). However, Boric Acid Lactose broth is considered less desirable than EC medium (Hall, 1964; Skinner and Njoku-Obi, 1957).

Temperature requirements are critical for the differentiation of E. coli (Hall, 1964). Eijkman (1904) originally used 46°C. This temperature was found to be too high (Hajna and Perry, 1939; Levine et al., 1934; Perry and Hajna, 1944; Skinner and Brown, 1934), inhibiting the growth of some strains of E. coli. Other workers suggested 43 and 44°C as the incubation temperature (Hajna and Perry, 1939; Levine et al., 1934; Walford, 1954). Most strains of Aerobacter aerogenes (E. aerogenes) produce gas from glucose, lactose and mannitol at 42°C, many at 44°C, but few at 46°C. The same temperature selection applies to A. cloacae (E. cloacae) and Citrobacter spp., however they do not produce gas from glucose or lactose at 46°C (Hajna and Perry, 1939). Hajna and Perry (1939) recommended the reduction of the incubation temperature to 45.5°C, but indicated that this could be lowered to 44°C for some foods (e.g. shellfish). As a result, a range of elevated incub-

ation temperatures has come into use. The International Committee on Microbiological Specifications for Foods (Thatcher and Clark, 1968) cited two methods: (i) the use of EC broth at 45.5° ± 0.2°C, and (ii) the predominantly European method, after Mackenzie et al. (1948), using Brilliant Green Lactose Bile 2% broth at 44° ± 0.1°C. Other elevated temperatures have been proposed such as EC medium at 44.5° ± 0.2°C for oysters (Kelly, 1960), and frozen foods (Fishbein and Surkiewicz, 1964). More recently, Mossel (1962) reported on the usefulness of two modifications of Eijkman's test for faecal contamination of foods, using the European method of Mackenzie et al. (1948) and Chapman's Lactose-Tergitol-Triphenyltetrazolium Chloride-Bromothymol Blue agar at 44°C. He reported that both methods were equally reliable.

Fishbein (1962) studied the effect of elevated temperature (44.5 to 46.5°C) on gas production by Aerobacter (Enterobacter) and E. coli strains. At 44.5°C, it was found that as many as 72% of Aerobacter cultures produced gas. Above 45.1°C there was a rapid drop in gas forming cultures by Aerobacter. At 45.5°C, only 2% of Aerobacter strains were gas positive, whereas 92% of the E. coli strains were gas positive. The E. coli strains maintained a high level of gas production up to 46°C. Above 46°C the incidence of gas production declined rapidly. The most resistant gas producers at elevated temperature were the E. coli type II (IMViC -+--) rather than the typical E. coli type I (++--).

Accordingly, Fishbein recommended 45.5° ± 0.1°C for the elevated temperature test. Subsequently, Fishbein and Surkiewicz (1964) studied the effect 44.5 and 45.5°C on the reliability of detecting faecal E. coli in frozen foods and nutmeats. They reported that 88% of 6,472 LST (Laury1 Sulfate Tryptose broth) tubes gave identical response at both temperatures. For those showing a difference in result at the two incubation temperatures, results at 45.5°C gave fewer false positives (threefold effect) but a 4% loss in recovery of E. coli over the lower temperature. Depending on the intention of the test, 44.5 or 45.5°C would be selected as the elevated incubation temperature.

Boric acid has also been extensively studied for its use as a selective agent. In 1934, Levine et al. studied the selectivity of boric acid on coliforms. In their study, boric acid medium was tested with a large number of Escherichia, Aerobacter and Citrobacter strains. They observed that at 43°C, Aerobacter was markedly inhibited, however 6.1% of the strains produced gas in 48h, and all Citrobacter strains were negative for gas production, whereas 97.3% of Escherichia strains produced gas. Vaughn et al., (1951) summarized the development of the boric acid medium. After they studied the effect of pH, bile, concentration of buffer and temperature of incubation on coliforms in boric acid medium, they developed a modified boric acid medium useful for selective isolation of E. coli. They observed that at 42.5 to 43.5°C, boric acid had little

effect on gas production by E. coli, but that gas production by Aerobacter (Enterobacter) and E. freundii (C. freundii) was inhibited. However, they also showed that spore-forming bacteria capable of fermenting lactose at 42.5 to 43.5°C were not significantly inhibited by boric acid.

Clark et al. (1957) studied the specificity of the boric acid medium and observed that coliforms of IMViC type ++-- gave a reasonably good percentage of positive reactions with Boric Acid Lactose broth at 43°C, but that a large number of positive reactions were also observed with IMViC types: --++, ++-+, and -+--. As a result, they did not recommend Boric Acid Lactose broth for rapid classification of the ++-- IMViC type.

The Health Protection Branch (HPB) of Canada Health and Welfare (Health Protection Branch, 1974) recommends the use of a 5-tube most probable number technique, using Lauryl Tryptose (LST) broth as presumptive test, Brilliant Green Bile 2% broth as confirmed test, and Levine EMB agar as completed test (Harrewijn et al., 1972). Proposed standards for ground meat specify limits of E. coli per gram. The HPB methods also recommend transfer of LST positive tubes to EC medium, incubated at 45° ± 0.2°C. To confirm faecal E. coli, the IMViC test for E. coli type I (++--) must be carried out. This is a laborious procedure, compared to the direct plating techniques recommended for dairy products, requiring 5 days for initial results, and a further 3 days to carry

out the IMViC tests.

Several rapid methods for the enumeration of coliforms or faecal E. coli in foods have been suggested, but they have not been thoroughly investigated, and their application to ground meats is not known. Abshire and Guthrie (1973) described a fluorescent antibody technique for the detection of faecal pollution (E. coli). Anderson and Baird-Parker (1975) used a direct plate method for enumeration of E. coli biotype I in foods. The method is based on the ability of E. coli to produce indole when growing at 44°C, on a cellulose acetate membrane, placed on a bile medium in a petri dish.

In a study of the significance of coliform bacteria in meats, therefore, it is important to consider not only the selective counts, but also the typical and atypical organisms that are included or excluded from the counts. In addition, Mossel and co-workers (1957; 1961; 1962) suggested the enumeration of Enterobacteriaceae instead of coliforms as an indicator of hygiene and sanitation. Enumeration of Enterobacteriaceae can be achieved by using glucose as the carbohydrate in a selective medium for enteric bacteria, or in the case of Violet Red Bile agar, by adding 1% glucose to the medium.

METHODOLOGY

I. Coliform and ENTEROBACTERIACEAE Counts in Ground Meats

Three qualities of ground beef and frozen and thawed pork sausage samples were purchased at retail stores and analyzed for bacteriological quality. An 11g sample of ground meat was homogenized with 99ml of 0.1% peptone water in a Waring Blendor jar, for 2 minutes at high speed as described by Al-Delaimy and Stiles (1975). Coliforms and Enterobacteriaceae were enumerated using different solid and liquid media.

A. Enumeration using the Direct Plating Technique

1. Violet Red Bile Agar (VRBA). Appropriate dilutions of the blended samples in 0.1% peptone water blanks were plated using the pour plate technique, with 15ml VRBA per plate and a 3-4ml overlayer (Difco Manual). The plates were incubated at 37°C for 24h. Differential counts of typical, bile precipitating colonies (Difco Manual) and atypical, non-bile precipitating colonies were recorded.

2. Mossel's Modified VRBA (VRBG). Difco VRBA with 1% glucose added was prepared and used as specified for VRBA above (Mossel et al., 1962).

B. Most Probable Number Technique

1. Presumptive Test for Coliforms. Appropriate dilutions were pipetted in triplicate into Difco Lauryl Tryptose broth (LST). Each tube contained an inverted Durham tube for reading gas production. The tubes were incubated at 37°C and read for gas production at 24h. Tubes that were gas negative at 24h were re-incubated and read at 48±2h.

2. Confirmed Test for Coliforms. A sterile loop was used to transfer cultures from all gas positive LST tubes to Difco Brilliant Green Bile 2% broth (BGB), containing an inverted Durham tube. Transfers were only made from LST tubes after 48h incubation. These BGB tubes were incubated at 37°C and examined for gas production at 24±2h. Gas negative tubes at 24h were re-incubated at 37°C and examined for gas production at 48±2h.

3. Completed Test for Coliforms. All BGB tubes were streaked onto Levine EMB agar (Difco) after 24h incubation. Gas negative BGB tubes were noted, by marking the EMB plates accordingly. Plates were incubated at 37°C and inspected after 24h for black colonies with a metallic sheen, or black centered (nucleated) colonies with transparent peripheries, or nucleated, mucoid colonies.

4. Elevated Temperature Test. LST positive tubes were inoculated into Difco EC broth, containing an inverted Durham tube and incubated at 45.5°C for 24h. Gas positive

tubes were noted at the end of each incubation period. This test was also carried out using direct inoculation of the sample dilutions into EC broth and similarly examined for gas production, as specified above.

The MPN was computed using the MPN index and 95% confidence limits for the combination of positive and negative tubes, using the 3-tube table reproduced from the American Public Health Association by Thatcher and Clark (1968).

II. Determination of the Types of ENTEROBACTERIACEAE Enumerated by the Different Techniques

A. Selection and Purification of Cultures

Cultures were selected from VRBA, VRBG and EMB plates. The subsurface colonies on VRBA and VRBG were selected and picked according to the amount of bile precipitation and other visual differences in colony morphology. Colonies with large, dark zones of bile precipitation (B++), faint zones of bile precipitation (B+) and those with no visible bile precipitation (B-) were selected, wherever possible, from plates representing each sample. Colonies were generally picked from plates with low densities to reduce chances of contamination by invisible or adjacent colonies in the medium.

Representatives of all colony types were picked from

EMB plates. These included typical colonies with a metallic sheen (MS+), and black-centered, "nucleated" colonies (NU+) and atypical mucoid colonies (MU+). In addition, non-coliform, pink to transparent, non-nucleated colonies (NU-) were selected.

The selected colonies were streaked onto Difco MacConkey agar (MA) to obtain isolated, surface colonies. A single, isolated colony on MA was selected and streaked onto a Nutrient agar (NA) plate to obtain a pure culture. The pure culture on NA was saved for biochemical tests. At the time that biochemical tests were carried out, purified cultures were transferred to Tryptic Soy broth (TSB) and frozen at -40°C for possible use in supplementary identification tests.

B. Identification of Cultures

1. Identification Tests

The methods selected to determine the identity of the selected cultures were based on the results presented in Method Development (p. 39).

(i) Screening tests: Gram stain for Gram negative rods; Oxidase test (Steel, 1961) for oxidase negative organisms; and acid from glucose.

(ii) Biochemical tests: Using the Minitek system, the following discs were included: arabinose, dulcitol,

inositol, raffinose and rhamnose for acid production; ONPG for β-galactosidase production; lysine and ornithine decarboxylation; phenylalanine deamination; H_2S and indole production; citrate and malonate as sole source of carbon.

Other tests using conventional tube techniques, included: gas from glucose, acid and gas from lactose using Phenol Red broth base (Difco) with 1% carbohydrate added (glucose broth was sterilized at 121°C for 15 min; a 10% lactose solution was filter sterilized using a 0.2μ millipore filter, and added aseptically to the sterile Phenol Red broth base); MR-VP (Difco) broth; and urea broth (Edwards and Ewing, 1972).

(iii) Supplementary and additional tests: LST (Lauryl Tryptose broth, Difco) for gas production; EC medium (Difco) incubated at 44.5 and 45.5°C for gas production; TSI (Triple Sugar Iron agar, Difco); Motility test using motility test medium (Difco). Supplemental tests necessary for further identification of the organisms were carried out using the conventional tube technique. The tests used were those recommended by "Roche Encise II".

(iv) Morphological studies: The selected cultures were re-plated onto VRBA, Mossel (VRBG) and EMB agars to confirm their morphology, and to check that the selected cultures had been tested, as opposed to adventitious "contaminants". In addition, the comparative morphology of the cultures on the selective media was determined.

2. Bacteriology

The Minitek plates were inoculated and incubated at 37°C as directed for the Minitek method. All carbohydrates and lysine, ornithine, and H_2S/indole discs were overlayed with 0.1ml sterile paraffin oil prior to incubation. Results were read after 18 to 24h incubation.

The other tests, except the MR-VP and EC broths were also incubated at 37°C. MR-VP was incubated at 30°C for 48h for the Voges-Proskauer reaction and for 5 days for the Methyl Red test. EC broths were incubated at 44.5 and 45.5°C ± 0.05°C in circulating, constant temperature water baths for 24 and 48h. Glucose, lactose, LST and TSI were incubated and read at 24 and 48h. TSI was only checked for delayed H_2S production at 48h. Motility test agar was read at 24h. Urea production was read at 24h, 48h, 4 days and 7 days.

3. Tests used to Identify Cultures

Cultures were identified using the Roche Encise II characteristics: acid and gas from glucose, lysine ornithine, H_2S/indole, lactose, dulcitol, phenylalanine, urease and citrate. The supplementary tests recommended by Roche Encise II were carried out, if necessary.

METHOD DEVELOPMENT

Several systems are available for identifying Enterobacteriaceae. Apart from the conventional methods of Cowan and Steel (1968) and Edwards and Ewing (1972) there are commercially prepared identification kits, e.g.
Auxotab (Wilson Diagnostics, Inc., 3 Science Rd., Glenwood, Ill., U.S.A.);
Enterotube (Roche Diagnostics, Division of Hoffmann-La Roche Inc., Nutley, New Jersey, U.S.A.);
the R/B system (Diagnostic Research Inc., Roslyn, N.Y., U.S.A.); and
the Minitek system (BBL, Division of Becton, Dickinson and Co., Mississauga, Ont., Canada).

The usefulness and accuracy of these commercial systems has been extensively studied, by comparison to the conventional methods (Blazevic et al., 1973; Hansen et al., 1974; Leers and Arthurs, 1973; Nord et al., 1974; Rhoden et al., 1973a,b; Robertson and MacLowry, 1974; Rosner, 1973; Tomfohrde et al., 1973).

Both the R/B and Enterotube systems are oriented to medical bacteriology for identification of human enteric isolates. They are based on a limited range of biochemical criteria, and lack versatility for use with the broader spectrum of Enterobacteriaceae that might be present in foods. The Minitek system, on the other hand, allows greater versatility in selection of biochemical criteria to be

tested and it is cheaper.

I. Comparison of Identification Systems

An initial study of 17 cultures (10 known and 7 unknown) was carried out to compare the performance of the conventional, Enterotube, R/B and Minitek systems. The 10 known cultures were coded and treated as "unknowns" to eliminate researcher bias in the identification of the cultures. The criteria used in the Minitek system were based on the system used by Hansen et al. (1974). The objective of this preliminary study was to determine the convenience, ease of interpretation and the accuracy of the results using each system.

The results of the R/B system were difficult to interpret, and with the results obtained, the cultures could not be identified with any degree of reliability. The identification of the cultures by the other systems is shown in Appendix A.

The known cultures could be identified more reliably than the unknown cultures. In general, positive identification was obtained for known cultures, but not for the unknown cultures, even with the complete conventional method. In some cases, the different systems resulted in different identities being assigned to the same cultures. This resulted from discrepancies in the results of the biochemical tests between systems. The principal

discrepancies with the Enterotube technique resulted from false positive indole tests, false negative citrate and urease tests. In the Minitek system, the principal discrepancies occurred in H_2S production, gas from glucose, unreliable urease, and false negative citrate tests.

The conventional technique was too laborious, especially with limited media preparation facilities. Furthermore, the conventional technique did not result in the definite identification of all cultures. Enterotube and Minitek were both convenient, but some difficulty was experienced in interpreting the tests which lead in part, to the discrepancies between the systems. Supplementary tests were required for both the Enterotube and Minitek systems to complete the identification. It was not known whether these discrepancies were due to deficiencies in the system, or incorrect interpretation of the results. Furthermore, the "known" cultures used in this study were not "type" cultures, so the need for supplementary tests might be due to the "known" cultures being atypical.

II. Comparison of Enterotube and Modified Minitek System to Identify Known Cultures

A second preliminary test was carried out using ATCC type cultures[1], referred to as designated strains in the 8th

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[1] American Type Culture Collection, 12301, Parklawn Drive, Rockville, Maryland 20852, U.S.A.

edition of Bergey's Manual (1974). The objectives of this test were to determine the accuracy of interpreting the Enterotube tests; the influence of incubation temperature on the Minitek results (for those cultures with recommended growth temperature of 30°C); and the ability to reduce the number of supplementary tests using the Minitek system.

In this trial, the tests used in the modified Minitek system were based on the flow diagrams in Figures 1-4. The minitek discs included: arabinose, citrate, H_2S/indole, inositol, lysine, malonate, ONPG, ornithine, phenylalanine, raffinose, rhamnose and urea. It was intended that the modified Minitek system should include all of the Enterotube tests, except dulcitol. In addition to the Minitek discs, glucose and lactose fermentation in phenol red broth (Difco Manual), H_2S on Triple Sugar Iron (TSI) agar (Edwards and Ewing, 1972; Difco Manual), urease activity in 3 separate media (Christensen, 1946; Vuye and Pijck, 1973), and motility in Motility Test medium (Difco), were tested.

The identity of the cultures is shown in Appendix B. Considerable discrepancy occurred in the reading of the Enterotube by the two laboratories, and between the Minitek as well as Enterotube systems. The interpretation of the Minitek results was based on a specially developed computer program. The combination of Minitek technique and the computer program gave an accurate identification of the cultures without additional supplementary tests, especially

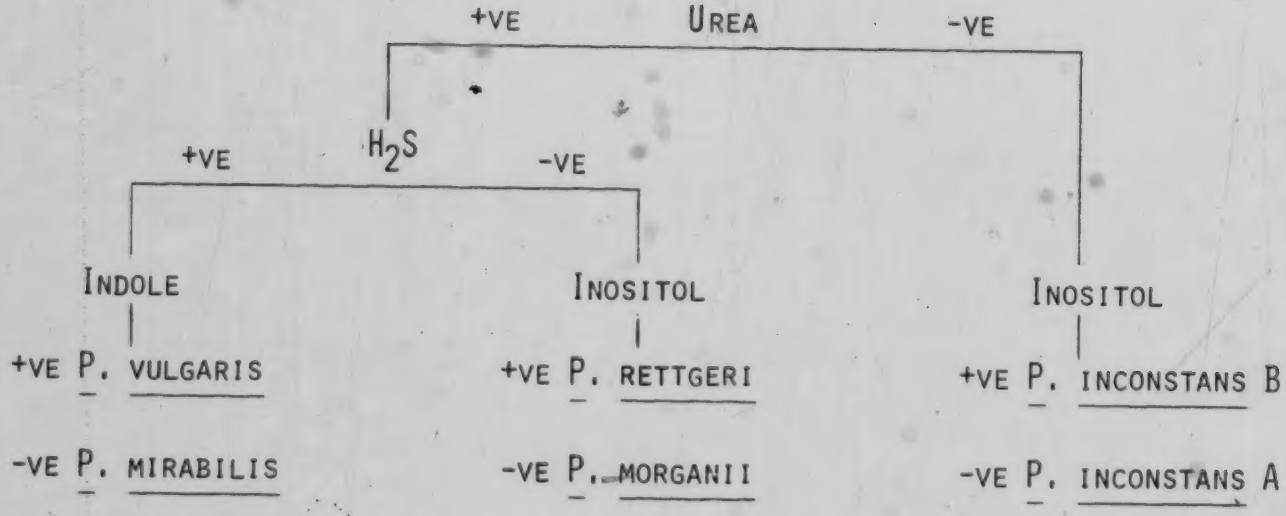

Figure 1. Flow diagram illustrating the tests used to differentiate the phenylalanine deaminase producing Enterobacteriaceae

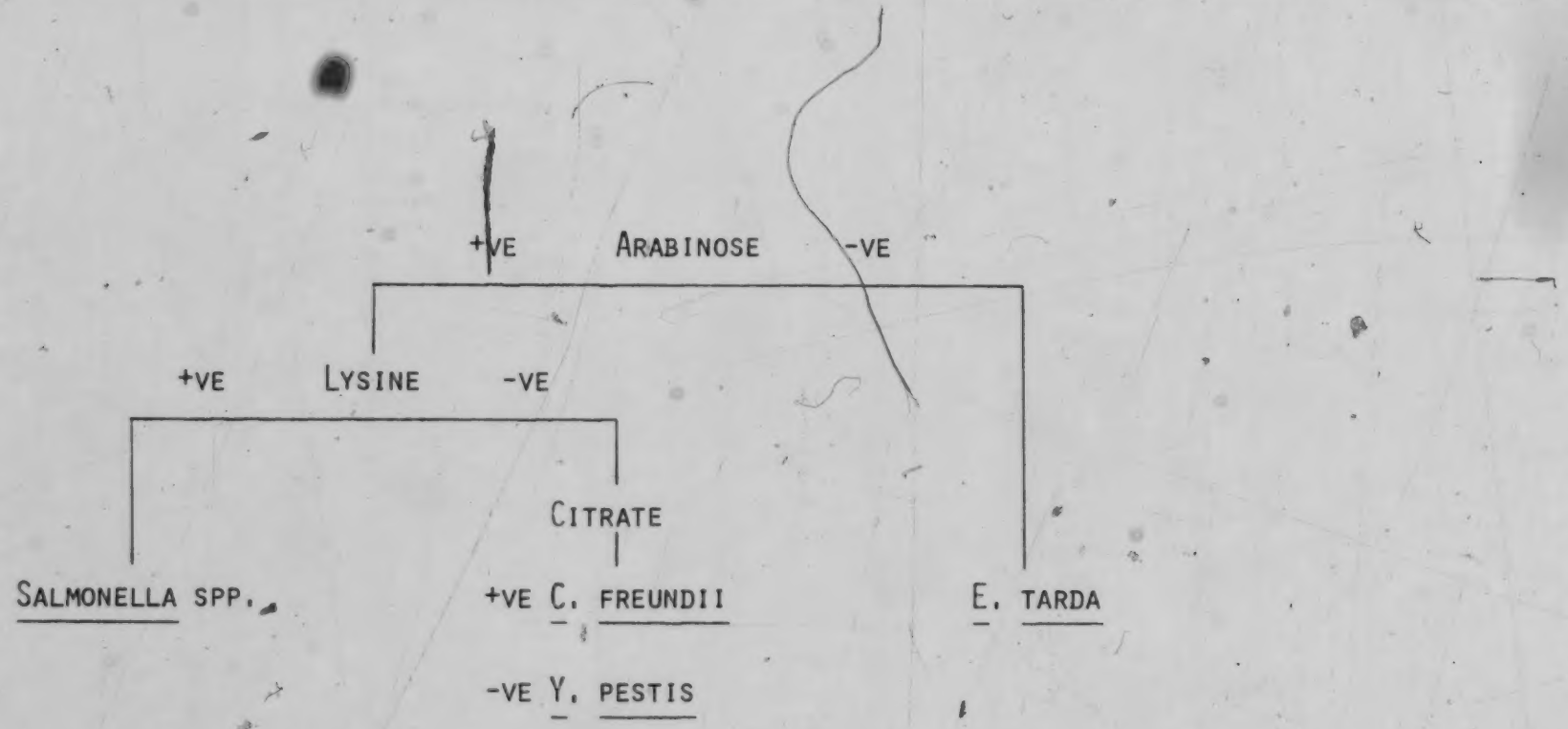

Figure 2. Flow diagram illustrating the tests used to differentiate the phenylalanine deaminase lacking Enterobacteriaceae which produce H_2S

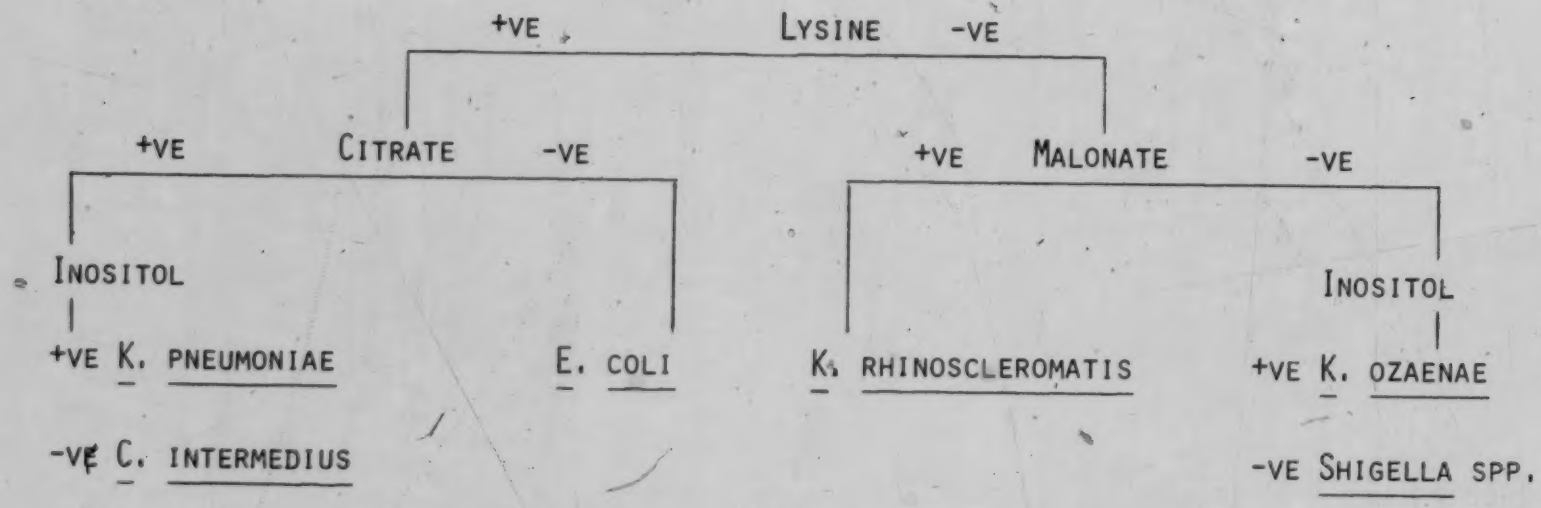

Figure 3. Flow diagram illustrating the tests used to differentiate phenylalanine deaminase lacking, indole positive and H_2S negative Enterobacteriaceae

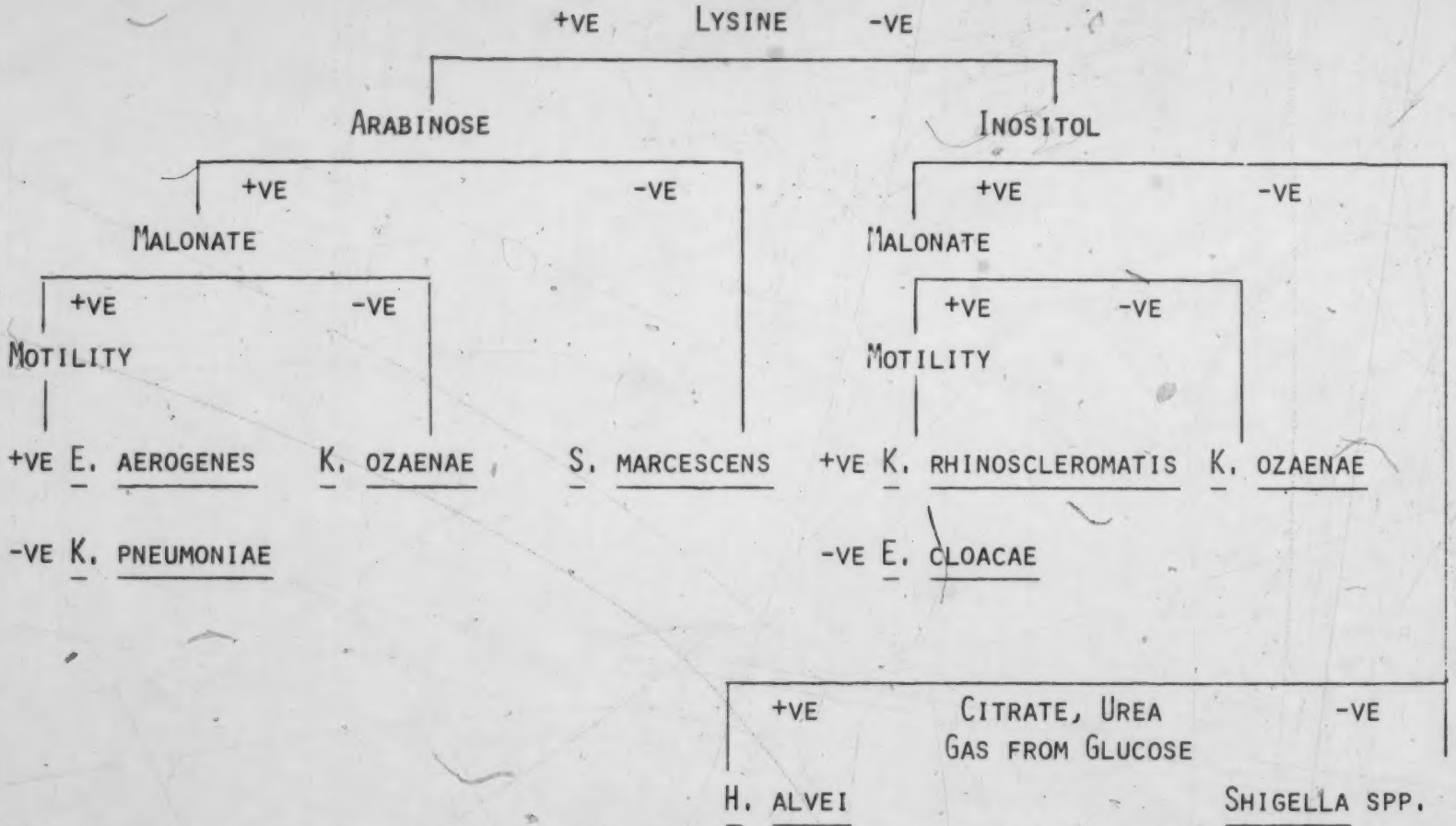

Figure 4. Flow diagram illustrating the tests used to differentiate the phenylalanine deaminase-lacking, indole and H_2S negative Enterobacteriaceae

at the genus level. The Enterotube results generally required supplementary tests to identify the cultures. Occasionally, the Enterotube resulted in incorrect identification for the known ATCC cultures. It was therefore decided to proceed with the modified Minitek and the computer program for identification of the unknown organisms. In the previous preliminary test, difficulty was reported in identifying the unknown cultures. The computer program will therefore be tested on the first series of unknown cultures (approximately 230) to test its ability to give a definite identification.

For the organisms grown and tested at 30 and 37°C, there were some minor differences in the test results, but not significant enough to affect the final identification. As a result 37°C was considered an appropriate incubation temperature, even for those cultures with recommended growth temperatures of 30°C.

III. Evaluation of Computer Program to Identify Unknown Cultures

The computer program developed to identify the unknown cultures consisted of a matrix of biochemical characteristics of known cultures (based on 8th edition of Bergey's Manual and the results for the ATCC type cultures). The program matched the unknown cultures with the organisms in the matrix, according to their biochemical characteristics.

The program determined the 6 highest matching organisms and the percentage match. No allowance was made for weighting of the importance of the different tests in the analysis.

The first 230 organisms tested by the modified Minitek technique were subjected to computer analysis. Although some cultures could be identified with a high degree of confidence, many of the cultures could not be given a positive identification, because more than one organism had the same percentage match. Furthermore, over 20% of the unknown cultures were identified as most probably being Shigella spp. However, Shigella spp. are not expected to be present in food (Bergey's Manual, 1974), and many of these suspected Shigella were motile. The difficulty with this program appeared to be that the various identifying characteristics were neither weighted nor exclusive.

The same known and unknown cultures were checked against the Roche Encise II identification scoring system. Because dulcitol had not been included in the modified Minitek system at this time, all dulcitol results were scored as negative, since this was the most likely test result (Bergey's Manual, 1974). The Roche Encise II system gave accurate identification of the ATCC type cultures, and reduced the number of doubtful or unidentifiable cultures for the unknowns. For example, most of the suspected Shigella spp. were identified as other organisms.

The result of this analysis indicated that the modified

Minitek method with Roche Encise II would be the best method for identifying the unknown cultures. The additional results obtained could then be used as supplementary tests. However, even with this technique, additional supplementary tests might be necessary, but the incidence of these tests was not sufficient to add any tests besides dulcitol to the modified Minitek technique.

IV. Selection of Biochemical Methods

The results with the Minitek discs were compared to the results of the conventional tube methods. In general, good agreement was observed between these results, except for urea and H_2S/indole. The main difficulty with the urea disc was the indefinite colour of the positive disc reaction. The Christensen's urea agar slant technique (Christensen, 1946) was first compared with the disc method. However, this agar slant method also gave intermediate colour reactions and was more difficult to prepare and inoculate than a broth. Therefore, Christensen's urea broth (Edwards and Ewing, 1974) was compared with the disc method for a large number of cultures. This method was more satisfactory and enabled delayed urease production, notably by some Klebsiella spp. (Edwards and Ewing, 1972), to be detected. In comparison, the Minitek urea discs were totally unreliable, resulting in numerous false positive and negative results.

RESULTS

I. Distribution of E. coli, Coliform and ENTEROBACTERIACEAE Counts

A total of 188 samples were analyzed using direct plating (VRBA and VRBG) and the MPN techniques (LST, BGB, EMB and EC). The distribution of coliform, Enterobacteriaceae and E. coli counts observed for the ground beef samples is shown in Table 7. Of the 169 ground beef samples, 104 (62.3%) samples had counts less than 100 E. coli per gram and 18 (10.8%) samples had counts exceeding 500 per gram. Similarly a total of 19 (79.2%) frozen pork sausage samples had counts below 100 E. coli per gram, and some of them exceeded 500 per gram. The only pork sausage samples that exceeded 500 E. coli per gram were those that had been thawed prior to sale.

II. Comparison of Counting Techniques

A. Differences between Sample Types

A $\log_{10}$ transformation of the data was performed and an analysis of variance[1] of the log transformed data was carried out. The analysis of variance results, shown in Table 8, indicate that although there is a statistically

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[1] ANOVAR program from SPSS. Computing Services, University of Alberta, Edmonton.

Table 7. Distribution of *Enterobacteriaceae* counts of ground beef

	Order of Counts per gram*					
	10^0	10^1	10^2	10^3	10^4	10^5
MPN TECHNIQUE	Number of Samples in each category:					
Presumptive coliforms	0	20	68	55	18	8
Confirmed coliforms	1	28	66	56	13	5
Completed coliforms	13	47	65	35	8	0
Faecal *E. coli*	28	76	44	15	1	0
DIRECT PLATING						
VRBA - Total	1	17	62	65	20	4
- Bile ppt	4	51	65	43	8	0
VRBG - Total	0	10	61	61	24	5
- Bile ppt	1	14	64	58	19	5

* 10^0 -- count < 10 per gram
10^1 -- count ≥ 10 < 100 per gram

Table 8. Statistical analysis of the E. coli, coliform, and Enterobacteriaceae counts on raw ground meats

1. Analysis of variance to test for significant differences between bacterial counts from meats and plating media.

Source[1]	Degrees of freedom	Mean squares	F ratio	Probability
A	4	18.954	4.524	0.002 **
Error (A)	179	4.189		
B	7	21.148	75.340	0.001 ***
A x B	28	0.284	1.013	0.447
Error (B)	1253	0.281		

2. Analysis of variance to test for differences between the 8 counts on pooled data.

Source	Degrees of freedom	Mean squares	F ratio	Probability
Sample Error (S(within))	183	4.614		
B	7	39.465	138.99	0.001 ***
B x S(within)	1281	0.284		

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[1] A: All samples (i.e. high, low and economy ground beef, frozen and thawed pork sausages).
B: Differential media counts (i.e. VRBA-T, VRBA-P, VRBG-T, VRBG-P, LST, BGB, EMB and EC).

3. Duncan's Multiple Range test.

Means in increasing order

EC	EMB	VRBA-P	BGB	LST	VRBG-P	VRBA-T	VRBG-T
1.704	2.342	2.370	2.754	2.926	2.932	2.949	3.076

AT 5% level of confidence[2]

EC	EMB	VRBA-P	BGB	LST	VRBG-P	VRBA-T	VRBG-T
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[2] The counts under the common line are not significantly different

significant difference between products, there is no interaction effect between product and method of enumeration (plating media). Since the objective of this part of the study is to compare the counts obtained on the different selective media, the differences between product types are not considered. Because of the lack of interaction effects between products and media, differences between media can be studied from these data (see below).

B. Differences between Media

The analysis shown in Table 8 indicates a significant difference between media ($p=0.001$). Duncan's Multiple Range test[1] based on the description of the test by Steel and Torrie (1960), was used to study media differences. At the 5% level of significance, the media could be separated into 5 separate groups, as shown in Table 8 (part 3): (i) The total count on Mossel's Enterobacteriaceae medium (VRBG-T), which was significantly higher than any of the other 7 counts. (ii) The presumptive coliform (LST) count, the bile precipitating colony count on Mossel's medium (VRBG-P) and the total count on Violet Red Bile agar (VRBA-T), which were similar. (iii) The confirmed coliform (BGB) count, which was significantly greater than the bile precipitating coliform count on Violet Red Bile agar (VRBA-P) and the completed coliform (EMB) count. (iv) The VRBA-P and EMB counts, which

[1] APL-DUNLN program. Computing Services, University of Alberta, Edmonton.

were similar, but significantly greater than (v) the E. coli (EC) count.

C. Correlation between Different Counts

Pearson's correlation coefficients[1] were calculated to test the linear relationship between the 8 counts. The results are presented in Table 9. All correlations between counts were highly significant (p=0.001). For ease of presentation, the results are considered as 3 groups.

(a) Direct plating media. The correlations between these counts were high, ranging from 0.77 to 0.97. The lowest correlation coefficients in this group were obtained for the Violet Red Bile agar, bile precipitating count (VRBA-P) with counts on the other media. On the other hand, VRBA-T, VRBG-T and VRBG-P were highly correlated, with greater than 80% predictability between the counts.

(b) MPN techniques. The correlation coefficients between the MPN coliform counts ranged from 0.72 to 0.92. The predictability of the BGB (confirmed) count from the LST (presumptive) count was 85%, however for the LST and BGB coliform tests the predictability of the EMB (completed) test was only 52 and 64%, respectively. The correlation coefficients between the MPN coliform counts (LST, BGB and EMB) and the elevated temperature E. coli count were low,

[1] Pearson's correlation SPSS program. Computing Services University of Alberta, Edmonton.

Table 9. Pearson's correlation coefficients between 8 different *Enterobacteriaceae*, coliform and *E. coli* counts*

	VRBA-T	VRBA-P	VRBG-T	VRBG-P	LST	BGB	EMB
VRBA-P	0.82						
VRBG-T	0.93	0.77					
VRBG-P	0.90	0.80	0.97				
LST	0.71	0.70	0.73	0.72			
BGB	0.67	0.70	0.69	0.72	0.92		
EMB	0.51	0.63	0.48	0.51	0.72	0.80	
EC	0.27	0.46	0.31	0.36	0.44	0.52	0.68

* VRBA Violet Red Bile agar
VRBG Mossel's *Enterobacteriaceae* medium (VRBA+1% glucose)

-T Total count of bile precipitating and non-bile precipitating colonies at 24h
-P Count of bile precipitating colonies at 24h

LST Lauryl Tryptose broth
BGB Brilliant Green Bile 2% broth
EMB Levine EMB agar
EC EC broth at 44.5°C

resulting in poor predictability of the E. coli count.

(c) Direct plating and MPN techniques. The strongest relationships exist between the LST and BGB counts and the direct plate counts, but only 40 to 50% of the count on the direct plating media could be predicted from the MPN counts. The relationship between the EMB (completed) coliform and the EC (E. coli) counts and the direct plating media was much less.

D. Effect of Temperature and Incubation Time on VRBA and VRBG Counts.

It was observed that both during refrigeration or on plates held at room temperature, more non-bile precipitating (B-) colonies grew on the VRBA and VRBG plates. Extended incubation at 37°C also resulted in higher numbers of B- colonies appearing on the plates. A fixed time-temperature of incubation, for example 37°C for 24±2h, was necessary for consistent results to be obtained.

E. EC Test

1. Comparison of the Direct and Indirect EC Tests

A total of 54 samples were analyzed by the direct and indirect methods in EC medium at 44.5°C for 24 and 48h. Log_{10} transformed data were compared using a t-test[1] which

gave a highly significant difference between the two counts ($p<0.01$). The mean of the counts for the direct test was 7.4×10^1/g compared to 1.4×10^3/g for the indirect test.

2. Comparison of EC Tests at 44.5 and 45.5°C

The 1,036 Enterobacteriaceae isolates from ground meats were inoculated into duplicate sets of EC broth and incubated at 44.5 and 45.5°C. The results for gas production are shown in Table 10, and may be summarized as follows:

(a) Of the 734 isolates that were gas negative after 48h incubation at 44.5°C, only one isolate produced gas at 45.5°C, resulting in 99.9% agreement in the tests.

(b) Of the 295 isolates that were gas positive after 24h incubation at 44.5°C, 7.8% (23 isolates) exhibited delayed gas production at 45.5°C, and 1.4% (4 isolates) failed to produce gas after incubation at 45.5°C for 48h. The 23 isolates giving delayed gas production at 45.5°C included 22 E. coli I and one E. agglomerans. The isolates that were gas negative with extended incubation at 45.5°C, included two E. coli I, a K. pneumoniae and an E. cloacae.

(c) There were only 7 delayed gas producers at 44.5°C, and three of these failed to produce gas after 48h at 45.5°C. None of these isolates were E. coli I.

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[1] t-test program of the SPSS. Computing Services, University of Alberta, Edmonton

Table 10. Relationship between elevated temperature tests in EC broth at 44.5°C amd 45.5°C

Gas produced in EC at 44.5°C	Gas produced in EC AT 45.5°C (percent of cultures)			Number of cultures
	Positive 24h	Positive 48h	Negative 48h	
Positive 24h	90.8	7.8	1.4	295
Positive 48h	28.6	28.6	42.9	7
Negative 48h	0.0	0.1	99.9	734

III. Identification of ENTEROBACTERIACEAE Isolates

A. Confirmation of Colony Morphology

The colonies isolated from VRBA and VRBG plates for identification were described on the basis of the presence and amount of visible bile precipitation at the time of selection. The colonies on EMB were selected according to their morphology (see methodology p.35). Because of the chances of contamination in selecting the isolates, especially when picking sub-surface colonies from the direct plating media (VRBA and VRBG), it was necessary to check the colony types of the purified cultures on the selective media.

Changes in morphology were observed when VRBA isolates were re-plated onto VRBA (see Table 11). For the original B++ isolates, a few (4.4%) were B+ on re-plating, but others (17.6%) formed B- colonies. Similarly, changes in morphology of original B+ and B- colonies occurred when they were re-plated onto VRBA. There was little concern for changes between B++ and B+, but a change to B- suggested either a contaminant or a marked change in the culture during purification and storage. For B+ isolates, it was difficult to interpret the changes from B+ to B++, and B+ to B- for the re-plated cultures, because the conditions on the original plates (e.g. meat particles in the inoculum) could have influenced the appearance of the colonies. For B-

Table 11. Comparison of morphology of colonies originally isolated from Violet Red Bile agar (VRBA) with their morphology when re-plated on VRBA after purification*

Original Morphology on VRBA	Morphology on VRBA (re-plated) B++	B+	B-	Number of cultures
B++	77.9% (53)	4.4% (3)	17.6% (12)	68
B+	32.7% (36)	33.5% (39)	31.8% (35)	110
B-	4.5% (6)	14.4% (19)	81.1% (107)	132
Totals	95	31	154	310

Table 12. Comparison of morphology of colonies originally isolated from Violet Red Bile agar + 1% glucose (VRBG) with their morphology when re-plated on VRBG after purification*

Original morphology on VRBG	Morphology on VRBA (re-plated) B++	B+	B-	Number of cultures
B++	80.2% (109)	15.4% (21)	4.4% (6)	136
B+	38.8% (40)	50.5% (52)	10.7% (11)	103
B-	18.7% (15)	50.0% (40)	31.3% (25)	80
Totals	164	113	52	319

* B++ -- Large dark zone of bile precipitation
B+ -- faint zone of bile precipitation
B- -- no bile precipitation

isolates, those that gave B+ and B- colonies on re-plating were considered similar, but a change to B++ suggested either a contaminant or a marked change in the culture during laboratory handling and storage.

Data for VRBG isolates re-plated onto VRBG are shown in Table 12, and the same considerations applied.

The data for the isolates from EMB, re-plated to EMB agar, are shown in Table 13. Considerable changes occurred in colony morphology, however the chance of contamination, compared to VRBA and VRBG, was much less because the isolates were surface colonies. Considerable changes could be observed between original MS+ and NU+ isolates, in fact as many as 60.8% NU+ isolates became MS+ (nucleated, with metallic sheen) on re-plating. Such changes in morphology were most likely due to pleomorphism, which in the case of the non-coliform NU- colonies, could be of considerable importance to the interpretation of the coliform count on EMB.

B. Association of Colony Morphology with Types of Organisms

The distribution of organisms between colony types is shown in Tables 14-16. The description of the colony morphology in these tables refers to the morphology both when the isolates were originally selected and after re-plating (confirmed colony morphology). This was necessary

Table 13. Comparison of morphology of colonies originally isolated from EMB agar with their morphology when re-plated on EMB after purification*

Original morphology on EMB	Morphology on EMB (re-plated) MS+	NU+	NU-	MU+	Number of cultures
MS+	85.9% (128)	9.4% (14)	2.0% (3)	2.6% (4)	149
NU+	60.8% (76)	24.0% (30)	10.4% (13)	4.8% (6)	125
NU-	17.1% (14)	12.2% (10)	67.1% (55)	3.7% (3)	82
MU+	27.6% (16)	15.5% (9)	15.4% (9)	41.4% (24)	58
Totals	234	63	80	37	414

* MS+ -- metallic sheen, nucleated colony
NU+ -- nucleated (pink + black centre) colony
NU- -- non-nucleated colony
MU+ -- mucous with or without a nucleus

for a reliable association of organisms with colony morphology to be obtained.

1. Violet Red Bile agar: Coliform Medium

The organisms growing on VRBA were divided into 3 categories, based on the degree of bile precipitation. The identity of the organisms associated with the different colony types (B++, B+, B-) is shown in Table 14.

(a) B++ colonies

The main organisms were E. coli I (46%), aerogenic E. agglomerans (15%) and E. cloacae (15%). On re-plating, the E. coli I accounted for an even greater proportion of the organisms associated with this colony type (60%). E. cloacae remained important, but E. agglomerans decreased in relative importance. Changes on re-plating could be attributed primarily to E. coli I colonies that were originally B+ becoming B++.

(b) B+ colonies

The main organism associated with this group was E. coli I (30% of the original isolates) but this decreased to 14% on re-plating because so many of the E. coli I gave greater zones of bile precipitation when re-plated in pure culture on VRBA. The principal organisms in this group became S. liquefaciens (30%) and aerogenic E. agglomerans (21%).

Table 14. Identity and number of organisms associated with different colony types on Violet Red Bile agar (VRBA) from original isolates and for purified (re-plated) cultures*

Organism	Original Colony Type			Confirmed Colony Type		
	B++	B+	B-	B++	B+	B-
E. coli I	31	34	4	57	9	3
E. coli II	1	2	2	2	1	2
Other *E. coli*	0	1	1	0	1	1
C. freundii	3	6	8	8	4	5
K. pneumoniae	5	4	3	6	5	1
K. ozaenae	1	2	0	0	0	3
E. agglomerans						
aerogenic	10	20	30	3	13	44
anaerogenic	1	4	19	1	1	22
E. aerogenes	0	3	2	0	1	4
E. cloacae	10	5	6	11	2	8
E. hafniae	3	3	5	4	3	4
S. liquefaciens	2	23	39	2	18	44
S. marcescens	0	0	1	0	0	1
S. rubidaea	0	2	2	0	0	4
Y. enterocolitica	0	0	1	0	0	1
Unidentifiable	1	2	1	1	1	2
Oxidase positive	0	1	16	0	2	15
Total number	68	112	140	95	61	164

* See Table 11

(c) B- colonies

The main organisms associated with this group were S. liquefaciens, aerogenic and anaerogenic E. agglomerans and the non-Enterobacteriaceae. Of the Enterobacteriaceae isolated as B- colonies, S. liquefaciens and E. agglomerans accounted for 63% of the isolates.

2. Mossel's Violet Red Bile Glucose agar: ENTEROBACTERIACEAE medium

The organisms growing on VRBG were handled and analyzed as described for VRBA, and the data are shown in Table 15.

(a) B++ colonies

The main organisms isolated originally and confirmed by re-plating as B++ were E. coli I (24% and 31%) and S. liquefaciens (26% and 31%). Other B++ isolates included C. freundii, K. pneumoniae, E. agglomerans, E. cloacae and E. hafniae, these organisms on re-plating, accounted for 30% of the B++ colonies. The same trend observed on VRBA, where B+ colonies became B++ on re-plating was observed on VRBG.

(b) B+ colonies

The main organisms in this group were aerogenic and anaerogenic E. agglomerans. The relative importance of E. coli I and S. liquefaciens decreased as a result of the re-plating, and the change of B+ colonies to B++.

Table 15. Identity and number of organisms associated with different colony types on Mossel's Enterobacteriaceae medium (VRBG) for original isolates and for purified (re-plated) cultures*

Organism	Original Colony Type			Confirmed Colony Type		
	B++	B+	B-	B++	B+	B-
E. coli I	34	14	5	51	2	0
E. coli II	4	1	2	4	2	1
C. freundii	8	3	2	10	3	0
K. pneumoniae	9	1	2	11	1	0
K. ozaenae	2	3	1	3	2	1
E. agglomerans						
aerogenic	15	21	20	8	38	9
anaerogenic	9	14	20	1	25	16
E. aerogenes	0	5	3	0	5	3
E. cloacae	12	4	2	12	6	0
E. hafniae	5	6	3	7	5	2
S. liquefaciens	36	26	5	51	15	1
S. marcescens	1	0	1	1	1	0
S. rubidaea	0	0	2	0	0	2
Proteus spp.	0	0	2	0	0	2
Y. enterocolitica	0	0	2	0	0	2
Unidentifiable	1	1	3	2	3	0
Oxidase positive	3	7	14	1	2	20
Total number	139	106	89	164	112	58

* See Table 12

(c) B- colonies

The main organisms among the Enterobacteriaceae in this group were aerogenic and anaerogenic E. agglomerans. After re-plating, they accounted for 66% of the Enterobacteriaceae growing as B- colonies on VRBG. Of the re-plated organisms, 30% of the B- colonies were not Enterobacteriaceae.

3. Eosin Methylene Blue agar: Confirmed Coliforms by MPN Technique

The organisms growing on EMB were identified by 4 different colony types: metallic sheen (MS+), pink with black centre (nucleated, NU+), non-nucleated (NU-) and mucous (MU+). The data are shown in Table 16. MS+ and NU+ are considered typical coliform colonies. MU+ are atypical coliform colonies but NU- are considered non-coliform bacteria.

(a) MS+ colonies

The principal organism in this group, from original isolates and after re-plating, was E. coli (67 and 66%, respectively). C. freundii and K. pneumoniae accounted for 24% of the isolates producing metallic sheen on re-plating. Some E. agglomerans also produced a metallic sheen, but the colonies did not have typical dark centres and the metallic sheen was faint. The increased number of MS+ colonies on re-plating could be primarily attributed to NU+ colonies

Table 16. Identity and number of organisms associated with different colony types on Levine EMB agar from original isolates and for purified (re-plated) cultures*

Organism	Original Colony Type				Confirmed Colony Type			
	MS+	NU+	NU-	MU+	MS+	NU+	NU-	MU+
E. coli I	100	58	9	4	154	14	1	2
E. coli II	3	3	1	0	6	0	1	0
C. freundii	13	17	17	5	27	16	8	1
K. pneumoniae	19	16	6	20	29	17	4	11
K. ozaenae	1	3	3	5	5	1	4	2
E. agglomerans								
aerogenic	5	5	3	2	5	0	5	5
anaerogenic	0	1	1	0	0	0	2	0
E. aerogenes	1	0	1	0	0	0	1	1
E. cloacae	1	13	11	15	3	11	14	12
E. hafniae	1	1	5	0	3	0	4	0
S. liquefaciens	1	6	12	5	1	3	19	1
S. rubidaea	0	0	1	0	0	0	1	0
Unidentifiable	1	1	0	2	2	0	0	2
Oxidase positive	3	2	12	0	0	1	16	0
Total number	149	126	82	58	235	63	80	37

* See Table 13

producing metallic sheen. This suggested a pleomorphism in colony types on EMB agar.

(b) NU+ colonies

Because of the above-mentioned change from NU+ to MS+ on re-plating, the number of NU+ colonies decreased from 126 to 63. Of the re-plated NU+ colonies, E. coli I (22%), C. freundii (25%), K. pneumoniae (27%) and E. cloacae (17%) were the main organisms.

(c) NU- colonies

NU- colonies are considered non-coliforms in the MPN technique. However, some pleomorphism was observed between NU- (non-coliform colonies) and typical coliform type colonies. For the re-plated colonies, the principal organism types included S. liquefaciens (24%), E. cloacae (18%) and 20% non-Enterobacteriaceae organisms.

(d) MU+ colonies

The principal organisms in this group were K. pneumoniae and E. cloacae.

C. Interrelationships of Colony Morphology between the Selective Media

All of the isolates obtained in this study were plated onto all 3 of the selective media (VRBA, VRBG and EMB) to determine the interrelationships of their colony morphology.

Dilutions that gave between 30 and 50 colonies per plate were inoculated onto each medium. The plates were incubated at 37°C and observed after 24±2h. The results are shown in Tables 17-19.

On Mossel's VRBG medium (Table 18) most of the Enterobacteriaceae produced a zone of bile precipitation around the colonies, only 49 (4.8%) of the Enterobacteriaceae failed to produce a visible zone of bile precipitation. Of these, 71.4% were E. agglomerans, representing 17.2% of the E. agglomerans isolated in this study. Of the total B- colonies on VRBG, 31.2% were non-Enterobacteriaceae. In contrast on VRBA (Table 17) a total of 404 (39.7%) Enterobacteriaceae isolates grew as non-bile precipitating colonies. The principal organisms producing B- colonies on VRBA were E. agglomerans (41.3%), S. liquefaciens (28.0%), E. cloacae (7.4%), C. freundii (5.7%) and E. hafniae (4.4%). Of the non-Enterobacteriaceae B- isolates, 95.1% grew as B- colonies on VRBA, representing 8.8% of the total B- isolates on VRBA.

All of the recognised coliform type organisms (E. coli, C. freundii, K. pneumoniae, E. aerogenes and E. cloacae) formed B++ or B+ colonies on VRBA. However, 27.4% of C. freundii isolates, 71.4% of E. aerogenes and 38.0% of E. cloacae failed to produce a visible zone of bile precipitation. Some non-coliform type organisms produced visible zones of bile precipitation on VRBA, including 28.9% of S.

Table 17. Growth characteristics of *Enterobacteriaceae* isolates on Violet Red Bile agar (VRBA) *

Organism	Percent growing on VRBA as: B++	B+	B-	Total Number
E. coli I	89.7	9.3	0.7	288
E. coli II	65.0	10.0	25.0	20
C. freundii	31.0	41.7	27.4	84
K. pneumoniae	72.9	21.2	5.9	85
K. ozaenae	33.3	19.0	47.6	21
E. agglomerans				
aerogenic	8.4	17.6	74.0	131
anaerogenic	1.4	2.7	95.9	73
E. aerogenes	7.1	21.4	71.4	14
E. cloacae	48.1	13.9	38.0	79
E. hafniae	25.8	16.1	58.1	31
S. liquefaciens	3.8	25.1	71.1	159
S. rubidaea	14.3	0.0	85.7	7

* B++ -- large zone of bile precipitation
B+ -- small zone of bile precipitation
B- -- no bile precipitation

Table 18. Growth characteristics of *Enterobacteriaceae* isolates on Mossel's Violet Red Bile agar + 1% glucose (VRBG)*

Organism	Percent growing on VRBG as: B++	B+	B-	Total Number
E. coli I	95.1	4.5	0.3	288
E. coli II	70.0	25.0	5.0	20
C. freundii	90.5	9.5	0.0	84
K. pneumoniae	98.8	0.2	0.0	85
K. ozaenae	76.2	19.0	4.8	21
E. agglomerans				
aerogenic	25.4	63.8	10.8	131
anaerogenic	2.7	68.5	28.8	73
E. aerogenes	42.9	57.1	0.0	14
E. cloacae	87.3	12.7	0.0	79
E. hafniae	74.2	19.4	6.4	31
S. liquefaciens	74.2	25.2	0.6	159
S. rubidaea	28.6	28.6	42.8	7

* B++ -- large zone of bile precipitation
B+ -- small zone of bile precipitation
B- -- no bile precipitation

liquefaciens and 18.1% of E. agglomerans.

Typical coliform colonies on EMB are nucleated, with or without metallic sheen (MS+ and NU+), atypical mucous colonies (MU+) also occur, and non-coliform colonies are non-nucleated (NU-). The results of plating the Enterobacteriaceae isolates from this study onto EMB are shown in Table 19.

The principal organisms growing as MS+ and NU+ colonies on EMB included E. coli, C. freundii, K. pneumoniae and E. agglomerans. Of these, E. coli, C. freundii and K. pneumoniae represented typical coliforms. Some K. pneumoniae grew as atypical coliforms (MU+), however 3.8% of E. coli, 21.4% of C. freundii, and 10.6% of K. pneumoniae grew as non-coliform organisms (NU-) on EMB. The majority of E. agglomerans (68.8%) were NU-, but those that were MS+ (12.2%) could be distinguished from the coliform organisms producing metallic sheen. The principal NU- organisms were S. liquefaciens and E. agglomerans. The non-Enterobacteriaceae organisms generally grew as NU- colonies on EMB. Some of the cultures failed to grow within 24h when replated onto selective media. However, some grew on VRBG and VRBA as B- colonies and on EMB as NU- colonies when incubated for a further 24h at room temperature.

Table 19. Growth characteristics of *Enterobacteriaceae* isolates on Levine EMB agar

Organism	Percent growing on EMB as: MS+	NU+	NU-	MU+	Total Number
E. coli I	84.1	13.1	1.7	1.0	290
E. coli II	65.0	10.0	25.0	0.0	20
C. freundii	50.0	27.4	21.4	1.2	84
K. pneumoniae	44.7	28.2	10.6	16.5	85
K. ozaenae	28.6	4.8	57.1	9.5	21
E. agglomerans					
aerogenic	16.8	16.8	60.3	6.1	131
anaerogenic	4.0	12.2	83.8	0.0	74
E. aerogenes	0.0	14.3	78.6	7.1	14
E. cloacae	6.3	26.6	39.2	27.8	79
E. hafniae	21.9	15.6	62.5	0.0	32
S. liquefaciens	1.9	5.7	91.1	1.3	158
S. rubidaea	0.0	14.3	85.7	0.0	7

D. Growth Characteristics of ENTEROBACTERIACEAE Isolates in Lauryl Sulfate Tryptose broth (LST) and Lactose broth

All of the isolates from the 3 selective growth media were inoculated into LST and Lactose broths. The results indicate which of the Enterobacteriaceae isolated in this study would be screened out by these presumptive coliform tests. The results for LST broth are shown in Table 20. The organisms that were able to produce gas from LST were E. coli I (93.6%), E. coli II (75.0%), C. freundii (67.9%), K. pneumoniae (90.6%), aerogenic E. agglomerans (46.3%), and E. cloacae (79.7%). Only 15.7% of S. liquefaciens isolates produced gas in LST tubes.

The results for growth in Lactose broth are shown in Table 21. The majority of E. coli I, C. freundii and K. pneumoniae produced acid and gas within 24h. Additional incubation for 48h only increased the lactose positive E. coli I by 0.7%, C. freundii by 6.0% and K. pneumoniae by 4.7%. Anaerogenic E. coli were observed, but their numbers were not high, and only 1.0% of E. coli I were lactose negative. E. cloacae isolates were generally aerogenic, lactose fermenters (86.1%). Of the 15 E. aerogenes isolates, 7 were non-lactose fermenters, the lactose fermenters were either aerogenic or anaerogenic.

The majority of S. liquefaciens were either non-lactose fermenters (71.1%) or anaerogenic for lactose (5.0%). The

Table 20. Growth characteristics of *Enterobacteriaceae* isolates in Lauryl Sulphate Tryptose broth (LST)

Organism	Percent growing on LST as: Positive 24h	Positive 48h	Negative 48h	Total Number
E. coli I	93.6	0.7	5.7	296
E. coli II	75.0	0.0	25.0	20
C. freundii	67.9	16.7	15.5	84
K. pneumoniae	90.6	3.5	5.9	85
K. ozaenae	71.4	9.5	19.0	21
E. agglomerans				
aerogenic	46.2	28.0	25.8	132
anaerogenic	5.4	9.5	85.1	74
E. aerogenes	13.3	13.3	73.3	15
E. cloacae	79.7	6.3	13.9	79
E. hafniae	40.6	12.5	46.9	32
S. liquefaciens	15.7	3.8	80.5	159
S. rubidaea	14.3	0.0	85.7	7

Table 21. Growth characteristics of *Enterobacteriaceae* isolates in Lactose broth*

Organism	Percent growing in Lactose broth as: A+G+ 24h	A+G+ 48h	A+G- 48h	A-G- 48h	Total Number
E. coli I	94.3	0.7	4.1	1.0	296
E. coli II	70.0	5.0	15.0	10.0	20
C. freundii	83.3	6.0	4.8	6.0	84
K. pneumoniae	88.2	4.7	5.9	1.2	85
K. ozaenae	61.9	14.3	4.8	19.0	21
E. agglomerans					
aerogenic	47.7	16.7	7.6	28.0	132
anaerogenic	8.1	8.1	32.4	51.4	74
E. aerogenes	33.3	13.3	6.7	46.7	15
E. cloacae	74.7	11.4	0.0	13.9	79
E. hafniae	40.6	12.5	3.1	43.8	32
S. liquefaciens	12.6	11.3	5.0	71.1	159

* A+G+ -- Acid and Gas produced
A+G- -- Acid produced, but no gas
A-G- -- No Acid or Gas produced

data in Table 21 indicate that aerogenic E. agglomerans were primarily lactose fermenters (64.4%), whereas the anaerogenic E. agglomerans were lactose negative (51.4%), and a further 32.4% were anaerogenic for lactose.

The comparison of LST and Lactose data is shown in Table 22. Anaerogenic lactose fermenters in Lactose broth were also negative in LST. In LST, 18% of lactose negative isolates were gas positive. The majority of the organisms accounting for this difference were E. agglomerans and S. liquefaciens. Similarly, 23.6% of anaerogenic lactose fermenters gave LST positive results. There were only 17 of these isolates, and of these, 5 were E. agglomerans and 4 were C. freundii. The delayed lactose and LST negative isolates were primarily S. liquefaciens and Enterobacter spp.; whereas the delayed lactose and LST cultures were mainly E. agglomerans; and delayed lactose and delayed LST positive isolates were principally C. freundii and E. agglomerans. Lactose positive cultures consisted of 4.3% delayed LST and 5.2% LST negative cultures. The delayed LST cultures were primarily C. freundii and E. agglomerans, whereas the LST negative cultures were principally C. freundii, S. liquefaciens, E. agglomerans and E. hafniae.

Table 22. Comparison of growth characteristics of Enterobacteriaceae isolates in Lauryl Tryptose (LST) and Lactose broths

Lactose	Percent growing in LST as: Gas at 24h	Gas at 48h	no Gas at 48h	Total Number
Acid+gas at 24h	90.5	4.3	5.2	629
Acid+gas at 48h	33.3	26.9	39.7	78
Acid only at 48h	9.7	13.9	76.4	72
Negative at 48h	7.2	10.8	82.1	251

Underlined results indicate tests which have compatible results in both tests

Table 23. Occurrence of IMViC types within Enterobacteriaceae isolates with different Lauryl Tryptose broth (LST) and Lactose reactions

IMViC types	LST+	LST-	Lactose+	Lactose-
++--	294	24	295	19
-+--	50	26	55	25
+-++	37	2	38	0
-+-+	67	15	73	8
--+-	11	15	25	14
---+	30	30	33	21
--++	164	172	208	130

E. Relationship of ENTEROBACTERIACEAE Isolates with IMViC Types

The Enterobacteriaceae isolates have been considered on the basis of their lactose fermentation and production of gas in LST. The IMViC types associated with these Lactose and LST results are shown in Table 23. IMViC types that were represented by less than 10 cultures have been eliminated from the results. Of the 7 remaining IMViC types, LST and Lactose positive cultures were mainly IMViC ++-- and --++, while for LST and Lactose negative cultures the principal IMViC type was --++.

The IMViC tests have been used to type lactose positive Enterobacteriaceae (coliforms). For these isolates, the association between IMViC type and type of organism is shown in Table 24. The ++-- types were generally E. coli I, but a few other lactose organisms were also ++--. The -+-- organisms were E. coli II, but some C. freundii and other organisms including K. ozaenae, Enterobacter spp. and S. liquefaciens were also observed as -+--. For the lactose fermenting E. agglomerans and S. liquefaciens, the principal IMViC group was --++, but these organisms were also distributed across most of the other IMViC groups presented in Table 24.

The results of IMViC tests on lactose negative isolates are shown in Table 25. The 5 lactose negative E. coli gave typical IMViC reactions. Lactose negative E. agglomerans

Table 24. Relationship of lactose positive organisms and IMViC types

Organisms	IMViC types of lactose positive Enterobacteriaceae						
	++--	+-++	-+-+	-+--	--+-	---+	--++
E. coli I	290	-	-	-	-	-	-
E. coli II	-	-	-	18	-	-	-
C. freundii	-	-	51	18	-	7	1
K. pneumoniae	3	35	-	-	-	2	31
K. ozaenae	-	-	3	3	-	9	1
E. agglomerans							
aerogenic	5	2	8	5	13	6	51
anaerogenic	-	2	1	4	3	6	17
E. aerogenes	-	-	-	-	-	-	6
E. cloacae	-	-	2	1	3	-	62
E. hafniae	1	-	3	2	3	4	5
S. liquefaciens	-	-	4	1	3	9	27

Table 25. Relationship of lactose negative organisms and IMViC types

Organisms	IMViC types of lactose negative Enterobacteriaceae					
	++--	-+-+	-+--	--+-	---+	--++
E. coli I	3	-	-	-	-	-
E. coli II	-	-	2	-	-	-
C. freundii	-	1	4	-	-	-
K. pneumoniae	-	-	-	-	-	1
K. ozaenae	-	-	3	-	-	-
E. agglomerans						
aerogenic	15	2	8	1	3	2
anaerogenic	1	2	8	6	5	5
E. aerogenes	-	-	-	-	-	7
E. cloacae	-	1	-	2	-	7
E. hafniae	-	1	-	3	1	7
S. liquefaciens	-	1	-	1	12	99
S. marcescens	-	-	-	1	-	1

included 15 that were ++-- and 16 -+--. Most of the lactose negative S. liquefaciens were --++.

Results for LST positive isolates were similar to the lactose positive results, shown in Table 24. For LST negative cultures the pattern was similar to that for Lactose negative isolates, but there was a greater number of E. coli that were ++-- (16 isolates) or -+-- (5 isolates).

The use of the elevated temperature test at 44.5°C in EC broth, in association with the IMViC tests, eliminated the organisms that were not E. coli in the type I (++--) and type II (-+--) groups, and will be discussed in the following section.

F. Association of ENTEROBACTERIACEAE with Elevated Temperature Test Results

The elevated temperature tests in EC broth were carried out at 44.5 and 45.5°C. The association of different organisms with the growth characteristics in EC broth at 44.5°C are shown in Table 26. The EC positive organisms at 44.5°C were primarily E. coli, however a small percentage (7.1%) of E. coli I were EC negative which would result in false negative results for 21 out of 296 isolates. There were only 20 -+-- isolates, but of these, 55% were EC positive and 45% EC negative. If elevated temperature (44.5°C) EC tests were used to identify faecal E. coli, false positives would result from E. coli II (-+--), K.

Table 26. Distribution of organisms with different growth characteristics in EC broth at 44.5°C

Organism	EC at 44.5°C Percent growing as: Positive 24h	Positive 48h	Negative 48h
E. coli I	92.2	54.1	2.9
E. coli II	3.7	-	1.2
C. freundii	-	-	11.4
K. pneumoniae	(1.0)	14.3	11.0
K. ozaenae	-	14.3	2.7
E. agglomerans			
aerogenic	(1.0)	14.3	17.4
anaerogenic	-	-	10.1
E. aerogenes	-	-	2.0
E. cloacae	(0.3)	-	10.6
E. hafniae	1.0	-	3.9
S. liquefaciens	-	-	21.6
Unidentifiable	0.7	-	1.5
Others	-	-	3.5
Totals	295	7	734

Numbers in brackets have one (0.3%) of the cultures negative at 45.5°C
Numbers underlined were negative at 45.5°C

pneumoniae, E. agglomerans, E. cloacae and E. hafniae. If 45.5°C was selected for the elevated temperature test, some false positives would be eliminated, but false negatives would be increased by approximately 2.3%.

IV. Evaluation of Minitek Technique for Identification of ENTEROBACTERIACEAE from Meat Samples

In the biochemical tests to identify the isolates in this study, glucose and lactose fermentation were determined in Phenol Red broth base. Triple Sugar Iron (TSI) agar slants were also inoculated to detect H_2S production. However, acid and gas production from glucose and acid from lactose could also be read on the TSI slants. Examination of the glucose and lactose results by both methods revealed some differences, as shown in Table 27. Using the tube glucose and lactose results as references, 4.3% of the gas positive isolates failed to produce gas from glucose on TSI slants, whereas 42.1% of gas negative isolates produced gas on TSI. This involved 84 isolates out of 1,022 tested. Similarly, for isolates producing gas and/or acid from lactose by the tube method, 13.9% failed to produce acid from lactose on the TSI slants. Of the lactose negative isolates, 24.7% produced acid on TSI (false positives).

From the modified Minitek method (see Methodology p.36) carried out on the isolates in this study, the basic data necessary for identifying these cultures using the Roche

Table 27. Comparison of glucose and lactose fermentation in Triple Sugar Iron (TSI) slant and Phenol Red broth tubes

Phenol Red broth	TSI slants				Total Number
	GLUCOSE		LACTOSE		
	acid+gas	acid only	acid	no acid	
GLUCOSE acid+gas (at 24h)	96.2% (865)	3.8% (34)			899
acid+gas (at 48h)	68.8% (11)	31.2% (5)			16
acid only (at 48h)	42.1% (45)	57.9% (62)			107
LACTOSE acid+gas (24/48h)			87.4% (618)	12.6% (89)	707
acid only (at 48h)			73.6% (53)	26.4% (19)	72
no acid (at 48h)			24.7% (62)	75.3% (189)	251

Encisé II system could be obtained. This resulted in the identification of 326 out of the 1,039 Enterobacteriaceae isolates. The modified Minitek method included additional tests which enabled a further 506 cultures to be identified in the Roche Encise II system, leaving 207 cultures to be identified using supplementary tests.

Of the 506 cultures identified with tests from the modified Minitek technique, the tests were used with the following frequencies:

Voges-Proskauer 472
ONPG 118
Motility 234
Arabinose 181
Rhamnose 337
Inositol 109
Raffinose 102
Methyl Red 5
Malonate 5

With the supplementary tests recommended by the Roche Encise II system but not included in the modified Minitek technique used in this study, a further 194 isolates were identified. The supplementary tests used and their frequencies are given below:

Cellibiose 93
Salicin 82
Arginine 44
KCN 31
Jordan's tartrate 22
Adonitol 15
DNase 9
Mucate 4
Sucrose 3
Capsule stain 3

DISCUSSION and CONCLUSIONS

I. Coliform and ENTEROBACTERIACEAE Counts

The distribution of the coliform and Enterobacteriaceae counts in these products were such, that they included samples that fell within the proposed Canadian E. coli standard (Pivnick, et al., 1975), exceeded the lower limit of 100 per gram, and exceeded the upper limit of 500 per gram. Marked differences were noted between product types however, the absence of interaction effects between product and plating media allowed the signigicant difference between media to be examined from this analysis.

According to Mossel et al. (1962), and based on the definition of Enterobacteriaceae as glucose fermenting organisms (Bergey's Manual, 1974), the bile precipitating colonies on Mossel's VRBG medium should represent the Enterobacteriaceae count. However, some of the non-bile precipitating colonies on VRBG were Enterobacteriaceae. This was confirmed by the re-plated isolates. Although there was a significant difference at the 5% level, but not at the 1% level, between the total and bile precipitating counts on VRBG, there was less than a 2-fold difference between the means of these counts. Many of the B- isolates (68.8% of 78 isolates) were Enterobacteriaceae; as a result, using the bile precipitating VRBG count to represent the Enterobacteriaceae would result in some false negatives, whereas using

the total VRBG count would give some false positive results. However, the differences are not great and the total count might be the easiest, since differentiation between low levels of bile precipitation and non-bile precipitating colonies is sometimes questionable, and this difficulty would be avoided.

In comparison, the total count on VRBA was statistically similar to the bile precipitating count on VRBG. The total count on VRBA, therefore, also estimates Enterobacteriaceae. The B- colonies on VRBA were principally E. agglomerans and S. liquefaciens, however some were atypical coliform-type organisms. The bile precipitating isolates were lactose fermenting organisms that included not only the generally recognised coliform organisms (E. coli, E. aerogenes, E. cloacae, K. pneumoniae, and Citrobacter spp.), but also other Enteropacteriaceae not normally included with the "coliform" bacteria, such as E. agglomerans, E. hafniae, K. ozaenae and S. liquefaciens. As a result, the classification of the organisms typically expected in the coliform count (i.e. lactose fermenting Enterobacteriaceae) needs to be expanded to include the lactose fermenting strains of the organisms listed above. On the other hand, the bile precipitating count on VRBA does not represent all of the "coliforms" because of false negative (atypical) coliforms.

Since the total count on VRBA estimates Enterobact-

eriaceae and the bile precipitating count estimates lactose fermenting Enterobacteriaceae, the use of the differential counts on VRBA might be preferred because they give more information than Mossel's VRBG medium.

In the MPN technique, the statistically significant differences between the counts did not represent a large, practical difference. There was only a 4-fold difference between the means of the completed (EMB) and the presumptive (LST) counts. None-the-less, the LST count estimates total Enterobacteriaceae, whereas the EMB count estimates "coliform" bacteria, and is the equivalent of the bile precipitating count on VRBA. Goepfert (1976) in his study of the microbiology of raw ground beef follows Hajna and Perry's (1943) proposal of using LST counts to estimate coliforms. Many studies have indicated that false positives are included in the LST count and the results of this study corroborate this. The fact that the LST count was statistically similar to the Enterobacteriaceae (total VRBA and bile precipitating VRBG) counts, and statistically different from the completed coliform (EMB) count, suggests either the occurrence of false positives in LST or inhibition causing false negatives in BGB, and subsequently on EMB.

In this study, 18% of lactose negative organisms in Phenol Red Lactose broth produced gas in LST. This might be expected because LST promotes lactose fermentation by slow lactose fermenters and anaerogenic coliforms (Mallman and

Darby, 1941). However, the organisms growing in LST included a large number of non-typical "coliforms", including some strains of E. agglomerans and S. liquefaciens, yet the majority of these organisms do not produce gas in LST. E. agglomerans was seldom identified as an MS+ or NU+ isolate from EMB, despite the fact that some E. agglomerans isolates on re-plating onto EMB were shown to produce light coloured colonies with a faint metallic sheen. E. agglomerans and S. liquefaciens were observed primarily as NU- colonies on EMB, i.e. as non-coliform Enterobacteriaceae. To use the LST count as a coliform count, further study of the types of organisms producing gas in LST broth would be necessary.

The elevated temperature test in EC broth is used to indicate faecal E. coli or E. coli type I. Some workers recommend direct inoculation of the meat homogenate into EC broth instead of inoculation (indirect) from the presumptive coliform test. The results of this study indicated that there was a significant difference between the direct and indirect methods, in which the indirect method gave results 2-fold greater than the direct method. In relation to the proposed E. coli standard for ground beef (Pivnick et al., 1975), this represents a major difference in the result, and the recommendation of the Health Protection Branch for this test must be clearly specified.

The elevated temperature test may be carried out at different temperatures. The most common temperatures in use

today are 44.5 and 45.5°C (Thatcher and Clark, 1968). False positive results were observed at 44.5°C, whereas false negative results occurred at 45.5°C. Incubation for 48h as opposed to 24h was more important at 45.5°C than at 44.5°C. The 7 (2.6%) false positives that grew at 44.5 and 45.5° could be due to unusual biochemical characteristics of these organisms, allowing them to grow at these elevated temperatures, or causing them not to be identified as E. coli. In addition, 11 (55.0%) E. coli II isolates (IMViC type -+--) produced gas at elevated temperature. This confirms the observation that both E. coli types I and II can produce gas under these conditions of incubation (Fishbein, 1962; Fishbein and Surkiewicz, 1964). E. coli I (IMViC type ++--) is used as the criterion of faecal contamination by the Canadian Health Protection Branch. The IMViC test, is intended to form the basis for prosecution in terms of this standard.

The MPN technique outlined by the Health Protection Branch, used in conjunction with the IMViC tests, is reliable for detecting faecal E. coli in meats, with only a limited number of false negative results. But the MPN technique is time consuming, and while suitable for regulatory purposes, it is totally unsatisfactory for quality control purposes. Based on the correlation coefficients between the EC counts and the other enumerating or estimating techniques used in this study, none of the other tests were capable of giving a reliable estimate of

faecal E. coli. It seems unlikely that a reliable, quick test could be obtained because the biochemical characteristics of E. coli are very closely related to other Enterobacteriaceae (Bergey's Manual, 1974).

As a result, the use of VRBA and direct or indirect EC counts at 45.5°C may be considered as routine methods to estimate Enterobacteriaceae, coliform bacteria and faecal E. coli. The deficiencies in this proposal should be apparent from the results of this study, none-the-less this recommendation is made because results would generally be available in 24h (except for gas negative EC tubes which should be incubated for a further 24h to include delayed gas producers).

Using VRBA and the direct or indirect EC test gives the following information:

1. If the bile precipitating count (coliform bacteria) is less than 1000 per gram, then the sample will most likely fit within the proposed standards of faecal E. coli.[1]

2. If the gas positive tubes by direct EC at 45.5°C are below 50 per gram and not greater than 250 per gram the sample will probably fall within the lower and upper faecal E. coli limits of the proposed standard, respectively.

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[1] For 119 samples with VRBA counts <1,000 per gram, 72.3% were <100 faecal E. coli per gram, 93.3% were <500 per gram, and only 6.7% were >500 per gram. For 44 samples with VRBA counts >1000 per gram, 52.3% were <100 faecal E. coli per gram, 77.3% were <500 per gram and 22.7% were >500 per gram.

3. If the gas positive tubes by indirect EC at 45.5°C are below 100 per gram and not greater than 500 per gram, the sample will probably fall within the lower and upper limits of the proposed standard, respectively.

4. The total VRBA count could be used as an indicator of sanitary handling and proper temperature control of the meats.

The VRBA count must be carried out at the specified temperature for 24±2h, to limit growth of non-Enterobacteriaceae, non-bile precipitating colonies (B-) on the plates. If 45.5°C is selected for the EC tests, fewer false positive results would occur, but false negatives would be observed at 24h. Extended incubation, for an additional 24h, reduces the false negative results. Conversely, incubation at 44.5°C reduces the need for extended incubation but false positive results will increase.

The use of E. coli as an indicator of faecal contamination of ground beef has been criticized by several workers (Hill, 1975; Goepfert, 1976). They suggested that contaminating E. coli could come from the intestine or hide of animals at slaughter. Furthermore, it is considered that E. coli could enter the food indirectly, from equipment. Goepfert (1976) claimed that E. coli thrive both in and out of the intestine. This contradicts the established premise of water bacteriologists that E. coli do not survive well in

an unfavourable environment. However, there can be little doubt that E. coli survive and grow, even at relatively low temperatures in ground meats (Al-Delaimy and Stiles, 1975). The purpose of identifying E. coli in foods is to indicate the possibility that intestinal pathogens might also be present. As such, the presence of E. coli of faecal origin in a food, by direct or indirect contamination, could indicate that intestinal pathogens are also present.

Mossel and co-workers (Buttiaux and Mossel, 1961; Drion and Mossel, 1972; Mossel, 1957;Mossel et al., 1962; Mossel et al.,1963) proposed the use of Enterobacteriaceae as indicators of sanitation and hygiene in foods. Not many of the other faecal Enterobacteriaceae referred to by Mossel, such as Proteus spp. and Klebsiella spp. (IMViC type ++++), were identified among the isolates of this study.[1] However, a large number of E. agglomerans and S. liquefaciens were detected in this study. E. agglomerans was previously Erwinia spp. of the Herbicola-Lathyri bacteria. With its description by Ewing and Fife (1972), the origin of this organism is described as "from a wide variety of environmental sources and from various kinds of specimens from man and lower animals." It is described as an "opportunist" organism that may cause human infections

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[1] Suspected Salmonellae obtained from Selenite Cystine enrichment, streaked onto Bismuth Sulfite and Brilliant Green agars, that were H S positive and lactose negative on TSI slants, were frequently shown to be Proteus spp., indicating that they were present in low numbers.

especially in the debilitated or young. The presence of E. agglomerans in a food, therefore, indicates lack of sanitation and/or hygiene. On the other hand, S. liquefaciens is reported as being found primarily in foods, and its implications in foods are not indicated (Ewing et al., 1973).

The sanitary and hygienic significance of these, and other Enterobacteriaceae, should be further studied to ascertain the importance of the total VRBA count in assessing sanitation and hygienic handling of meats. This is particularly important because the coliforms were significantly less than Enterobacteriaceae in meats, and the total VRBA count might be a better parameter for use in quality control. For the reliable interpretation of the significance of other Enterobacteriaceae in quality control, more information on the growth characteristics of these organisms is required.

II. Classification of Coliform Bacteria

Lactose fermentation is used as a screening test for coliform bacteria. However, atypical coliform (non-lactose fermenters or anaerogenic) bacteria were observed in this study. In the case of E. coli I, there were not many atypical strains (approximately 5% of 296 isolates). In contrast, the Enterobacter spp. (cloacae and aerogenes) usually considered as coliform bacteria had much larger

numbers of atypical strains (18% of 94 isolates). Lactose as the sole basis of classifying coliform bacteria results in many organisms expected to be included in the coliform count, being omitted. The use of lactose fermentation as the criterion for coliform bacteria became further complicated by the fact that S. liquefaciens and E. agglomerans are variable lactose fermenters (Ewing and Fife, 1972; Ewing et al., 1973).

IMViC tests were established for subdividing coliform bacteria into groups. This study revealed that the IMViC grouping was specific for E. coli, but not for other coliform bacteria. The non-lactose fermenting Enterobacteriaceae, typed by IMViC tests, were also found to be distributed across all IMViC types. Because of this, atypical coliforms cannot be classified by their IMViC reactions. Furthermore, the elevated temperature test data used in conjunction with the IMViC test data, refers only to the IMViC types ++-- or -+--, and most, or all, of these organisms are E. coli, especially if 45.5°C was used as the incubating temperature, instead of 44.5°C. As a result, the classification scheme proposed by the Coli-Aerogenes Sub-committee (Report, 1956) is only useful for screening out E. coli type I.

The Coli-Aerogenes Sub-committee (Report, 1956) also proposed the use of gelatin liquefaction in the classification of coliform bacteria. Gelatin liquefaction

would only be of value in distinguishing between K. aerogenes and K. cloacae, therefore, it was not used in this study. The use of an additional test such as gelatin liquefaction expands the tests used to classify the coliforms to the point where the isolates might as well be identified by Enterobacteriaceae identification kits.

III. Minitek Identification Technique

The Minitek technique enabled both typical and atypical Enterobacteriaceae isolated from meat samples to be identified. The limitations of other identification kits (Enterotube and R/B System) was shown in method development (p. 36). The limitation of the Enterotube criteria for these isolates was also shown when only 326 out of 1039 isolates from these meat samples could be identified by the Roche Encise II system, without supplementary tests.

The modified Minitek system used in this study enabled a further 506 cultures to be identified from the Roche Encise II system, indicating that the modified Minitek technique was more efficient than Enterotube. Based on the overall identification data it is recommended that for identification of Enterobacteriaceae from meats the following tests should be carried out:

1. Acid and gas from glucose and lactose
2. Acid from arabinose, cellibiose, dulcitol, inositol, raffinose, rhamnose, and salicin.

3. Utilization of citrate as a sole source of carbon
4. Production of acetyl methyl carbinol (Voges-Proskauer reaction)
5. Production of lysine and ornithine decarboxylases
6. Production of phenylalanine deaminase
7. Production of β-galactosidase (ONPG test)
8. Production of urease
9. Production of H_2S and indole
10. Growth on Triple Sugar Iron (TSI) slants.
11. Motility test.

All of these tests, except the TSI slants and Motility test can be carried out using Minitek discs. However, the limitations experienced with detection of gas production from glucose and lactose, the indefinite colour changes on urea discs[1] and the lack of agreement between results on the H_2S Minitek disc[2] compared to H_2S production on TSI slants, caused conventional tube media to be used for these tests in this study. Although the TSI slant is capable of giving acid and gas from glucose, and acid from lactose, in addition to H_2S production, the lack of agreement between TSI glucose and lactose results and the results by the conventional tube techniques prompted the use of conventional tube methods as the reference for this study.

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[1] Subsequent to this study, a new urea disc was developed by BBL Minitek to give a more clearly defined colour change in this test.

[2] Subsequent to this study, a new H_2S disc was developed by BBL Minitek to give improved performance in this test.

For the H_2S results, the difference between Minitek and TSI methods might be suspected since most descriptions of H_2S production are based on TSI slants (Bergey's Manual, 1974), and Vaughn and Levine (1936) reported that agar had a marked influence on H_2S production. The urease test in Christensen's urea broth (Edwards and Ewing, 1972) revealed many slow urease producers that required up to 7 days for a positive reaction. Slow urease production could account for the difficulty experienced with the Minitek urea test.

ONPG is not one of the recommended tests for the Roche Encise II system. The Roche Encise II system uses serological typing of Salmonella, however this was not used in this study and ONPG positive tests were used to screen out Salmonella. THe proposed tests for identification of Enterobacteriaceae from meats are outlined below:

Minitek discs

Phenylalanine
ONPG
Citrate
Malonate
Indole
Lysine
Ornithine
Arabinose
Cellibiose
Dulcitol
Inositol
Raffinose
Rhamnose
Salicin

Conventional Media

Glucose
Lactose
MR-VP

TSI
Urea
Motility

If other studies indicate a different range of Enterobacteriaceae, other biochemical tests might be necessary. Minitek allows this flexibility. In this study, the Roche Encise II system for identification of Enterobacteriaceae was used, however it would appear that a computerized system appropriate for the selected modified Minitek system should be developed.

SUMMARY

1. Statistically significant differences were observed between the different Enterobacteriaceae, coliform and E. coli counts obtained on the ground meats.

2. Mossel's Enterobacteriaceae medium (VRBG) enumerated Enterobacteriaceae, however some atypical Enterobacteriaceae that failed to prepicipitate the bile salts were observed on this medium.

3. The total count on VRBA and the bile precipitating count on VRBG were statistically similar, and estimate the total Enterobacteriaceae count.

4. The bile precipitating count on VRBA represents typical coliform bacteria, hence using VRBA total and bile precipitating counts gives more information than enumerating Enterobacteriaceae on VRBG.

5. Although there was no statistical difference between coliforms enumerated on VRBA and EMB, the organisms comprising the counts were different. Hence the lack of statistical difference might be fortuitous.

6. The direct plating techniques could not be used to estimate E. coli counts.

7. To confirm E. coli I, EC at elevated temperature (45.5°C) should be used to screen E. coli. VRBA and EC tests might be used to give more rapid, less laborious quality assurance information.

8. E. coli I was isolated from most samples in this study and represented 29% of the isolates. E. agglomerans and S. liquefaciens were frequently isolated and represented 21 and 16% of the isolate, respectively. However, the significance of these organisms in ground meats is not known.

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APPENDIX A. Identification of 10 Known and 7 Unknown *Enterobacteriaceae* Cultures using Different Identification Systems

Cultures	Conventional technique	Enterotube & Encise II[1]	Minitek technique
S. flexneri	*Shigella*	#2040	*Shigella*
K. pneunoniae	*K. pneumoniae*	#3423	*Klebsiella*
E. coli	*Escherichia*	#2060	*Escherichia*
Unknown	*S. liquefaciens*	#3200	*Serratia*
E. hafniae	*E. hafniae*	#3600	*E. hafniae*
P. vulgaris	*P. mirabilis*	*P. mirabilis*	*P. mirabilis*
Unknown	*Escherichia*	#2060	*Escherichia*
C. freundii	*C. freundii*	#3240	*Escherichia*
S. typhimurium	*Salmonella*	#3311	*E. cloacae*
Unknown	*Escherichia Shigella*	#3060	*Shigella*
Unknown	*E. cloacae*	#3020	*Shigella*
Unknown	Unidentifiable	#2070	*Shigella*
K. pneumoniae	*Klebsiella*	#2000	*Klebsiella*
Unknown	Unidentifiable	#2070	*Citrobacter*
Unknown	*K. pneumoniae*	*K. pneumoniae*	*Klebsiella*
S. marcescens	*S. marcescens*	#3600	*Serratia*
E. aerogenes	*E. aerogenes*	*E. aerogenes*	*Arizona*

[1] See addendum for probable identity of organisms, associated with these I.D. numbers in Encise II.

APPENDIX B. Results for Identification of ATCC Type Cultures

Cultures	Identity of cultures A[1]	B[1]	Modified Minitek
K. pneumoniae	#2425	#3427	K. pneumoniae
K. ozaenae	#2007	#2403	K. ozaenae
K. rhinoscleromatis	#2000	#2000	K. rhinoscleromatis
E. coli	#2620	#3630	E. coli
C. freundii	#2021	#2122	C. freundii
C. intermedius	#3021	#3101	C. intermedius
S. cholerae-suis	#2601	#3213	S. cholerae-suis
S. typhimurium	#3601	#3701	S. typhimurium
S. flexneri	#2000	#2000	Unidentifiable
P. vulgaris	#2105	#2147	P. vulgaris
P. morganii	#2201	#2207	P. mirabilis
E. tarda	#2600	#3740	E. tarda
S. marcescens	#2601	#2603	S. marcescens
E. cloacae	#3221	#3221	E. cloacae
E. aerogenes	#3601	#3621	E. aerogenes
H. alvei			
37°C	#2600	#3600	E. aerogenes
30°C	#2601	#3602	E. aerogenes
P. inconstans A			
37°C	#2005	#2006	P. inconstans A
30°C	#2005	#2006	P. inconstans
Y. enterocolitica	#2201	#2343	Shigella or Y. enterocolitica

[1] A = Enterotube result from Enterobacteriaceae laboratory, University of Alberta, Edmonton. The numbers are the identity number from the Roche Encise II (see Addendum).
B = Enterotube result from researcher's laboratory. The numbers are the identity number from the Roche Encise II (see Addendum).

Appendices A and B: Addendum

Encise II Identity #	Organism(s)	Reaction score
2000	identity not well defined	
2005	Anaerogenic E. agglomerans	0.9999
	P. vugaris	0.0001
2006	Anaerogenic E. agglomerans	o.8860
	P. rettgeri	0.0822
	P. vulgaris	0.0318
2007	Anaerogenic E. agglomerans	0.5103
	P. rettgeri	0.4887
	P. vulgaris	0.0010
2020	identity not well defined	
2021	S. rubidaea	0.6699
	Anaerogenic E. agglomerans	0.2729
	K. ozaenae	0.0555
	C. freundii	0.0016
2040	identity not well defined	
2060	identity not well defined	
2070	Alkalescens Dispar	0.9861
	Escherichia	0.0139
	C. freundii	0.0001
2105	P. vulgaris	1.0000
2122	C. freundii	1.0000
2147	P. vulgaris	1.0000
2201	identity not well defined	
2207	P. mirabilis	1.0000
2343	C. freundii	1.0000
2403	K. ozaenae	0.9989
	E. aerogenes	0.0011
2425	unidentifiable	
2600	identity not well defined	
2601	identity not well defined	

2603	S. marcescens	0.9451
	S. liquefaciens	0.0516
	K. ozaenae	0.0020
	E. aerogenes	0.0013
2620	E. aerogenes	0.4166
	S. liquefaciens	0.2902
	K. ozaenae	0.1817
	Escherichia	0.1114
3020	identity not well defined	
3021	identity not well defined	
3060	Escherichia	0.5436
	Aerogenic E. agglomerans	0.4558
	C. freundii	0.0006
3101	C. freundii	0.9906
	Salmonella spp.	0.0094
	H_2S +ve Escherichia	0.0001
3200	identity not well defined	
3213	E. cloacae	0.6660
	C. freundii	0.3340
3221	E. cloacae	0.6708
	S. liquefaciens	0.3116
	C. freundii	0.0099
	K. ozaenae	0.0078
3240	Escherichia	0.9970
	C. freundii	0.0030
3311	Salmonella	0.7140
	C. freundii	0.2859
	H_2S +ve Escherichia	0.0001
3423	K. pneumoniae	0.9615
	S. rubidaea	0.0258
	K. ozaenae	0.0072
	E. aerogenes	0.0055
3427	unidentifiable	
3600	identity not well defined	
3601	identity not well defined	
3602	identity not well defined	

3621	*E. aerogenes*	0.8564
	S. liquefaciens	0.0813
	E. hafniae	0.0317
	K. ozaenae	0.0006
3630	*Escherichia*	0.7494
	E. aerogenes	0.2506
3701	identity not well defined	
3740	*Edwardsiella*	0.9821
	H_2S +ve *Escherichia*	0.0179